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VOL. 42 DECEMBER, 1953 NO. 6

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

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Vol. 42, No. 6, December, 1953. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription price: United States, its possessions, Pan-American Countries, $12.00 per year; Canada, $13.00; Foreign, $13.00. Subscriptions to medical students, interns and residents, half-price. Entered as Second Class Matter October 8, 1915, at the Post Office at St. Louis, Missouri, under act of March 3, 1879. Printed in the U. S. A.

The Journal of Laboratory and Clinical Medicine

VOL. 42 DECEMBER, 1953 NO. 6

CLINICAL AND EXPERIMENTAL

THE UPTAKE IN VITRO OF RADIOACTIVE IRON BY AVIAN ERYTHROCYTES

W. N. JENSEN, M.D.,* HELEN ASHENBRUCKER, B.S., G. E. CARTWRIGHT, M.D., AND M. M. WINTROBE, M.D., PH.D.

SALT LAKE CITY, UTAH

SINCE Shemin, London, and Rittenberg[1, 2] demonstrated in 1948 that the nucleated red blood cells of the duck are capable of synthesizing heme in vitro, numerous studies have been performed by this means on various aspects of hemoglobin synthesis. The biosynthesis of protoporphyrin has received extensive study[3, 4] and investigations on the incorporation of various amino acids into globin have been carried out in several different laboratories.[5-8] The incorporation of iron into the protoporphyrin moiety has been investigated less extensively. Walsh and associates[9] have shown that human reticulocytes are able to assimilate iron in vitro for heme synthesis and that this uptake of iron may be used as an indicator of the rate of hemoglobin synthesis. Sharpe, Krishman, and Klein[10] found that an appreciable fraction of the radioiron added to duck blood enters erythrocytes during incubation and is recoverable in the heme fraction.

It seemed possible to us that measurement of iron uptake by red cells might be used as a means of assaying the degree of heme synthesis in vitro

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This investigation was supported in part by a research grant from the National Institutes of Health, U. S. Public Health Service, and in part by a contract (AT [11-1] -82), Project No. 6, between the University of Utah and the U. S. Atomic Energy Commission.

Received for publication, Aug. 3, 1953.

*Senior Fellow of the Damon Runyon Memorial Fund for Cancer Research.

under various experimental conditions. It was therefore decided to study factors influencing the movement of iron into duck erythrocytes and its distribution in these cells.

METHODS

Adult white Pekin ducks of either sex of approximately 2 kilograms of body weight were used in these experiments. The animals were fed mixed grains and greens ad libitum.

Blood was aspirated from either the jugular or wing vein into clean heparinized syringes. Six to twenty-five milliliters of whole blood were placed in cotton-stoppered Erlenmeyer flasks to which were added 2 mg. each of sodium benzyl penicillin and streptomycin calcium complex. The radioiron* and the nonradioactive iron were added as a solution of ferric chloride. The flasks were then incubated in a water bath at 37° C. and pH 7.4 and gently agitated constantly for varying periods of time.

The iron^{59} content of the whole blood, plasma, erythrocytes, and hemin was determined by the use of a vial-type gamma ray scintillation counter.† The plasma was separated by centrifugation. Erythrocytes were washed three times in cold isotonic saline. The washed erythrocytes were hemolyzed by the addition of saponin and distilled water. The solution of hemoglobin which contained red cell stroma and nuclei was then homogenized for five minutes in a Waring blendor. Crude hemin was isolated by the method of Fischer[11] and recrystallized and purified as outlined by Shemin and co-workers.[2]

Hemoglobin was determined by the oxyhemoglobin method with the aid of an Evelyn photoelectric colorimeter at wave length 540 mμ.[12] The instrument was standardized by the iron method. The total amount of heme present in a sample was calculated from the amount of hemoglobin present. The specific activity of the isolated hemin was determined and the total activity of the heme present in the original samples calculated. The results are expressed as the percentage of radioiron added or as the actual amount of iron transferred from one compartment to another. The latter was calculated by multiplying the total amount of iron in the plasma by the percentage of radioiron uptake into the indicated compartment.

Hematologic determinations were performed according to methods previously described.[12] Reticulocytes per 1,000 erythrocytes were enumerated. A modification[13] of the method of Barkan and Walker[14] was used for the determination of plasma iron content.

RESULTS

The Uptake of Radioiron in Vitro by Duck Erythrocytes.—To determine the rate and extent of the in vitro uptake of radioiron by duck erythrocytes, 120 ml. of blood (V.P.R.C., 40 ml./100 ml.; reticulocytes, 13 per cent; plasma iron concentration, 134 μg per cent) were withdrawn from a duck and divided into six 20 ml. aliquots. To each of the six flasks 0.0082 μc of iron^{59} was added. These were then incubated at 37° C. for 1, 2, 3, 6, 12, and 24 hours, respectively. The radioactivity in the plasma, erythrocytes, and hemin was then determined. The results of one of three such studies are presented in Fig. 1. As the iron^{59} disappeared from the plasma, it appeared in the washed erythrocytes. After six hours' incubation, 85 per cent of the iron^{59} was found in the erythrocytes and only 15 per cent remained in the plasma. Eighty-five per cent of the iron^{59} in the erythrocytes was found in the recrystallized hemin. The remainder (15 per cent) in the erythrocytes has been termed the "non-heme red blood cell iron."

*Obtained by allocation from the Isotopes Division of the U. S. Atomic Energy Commission, Oak Ridge, Tenn.

†The scintillation counter was made by H. O. Anger, Berkeley, California, and has a counting efficiency for iron^{59} of 21 per cent.

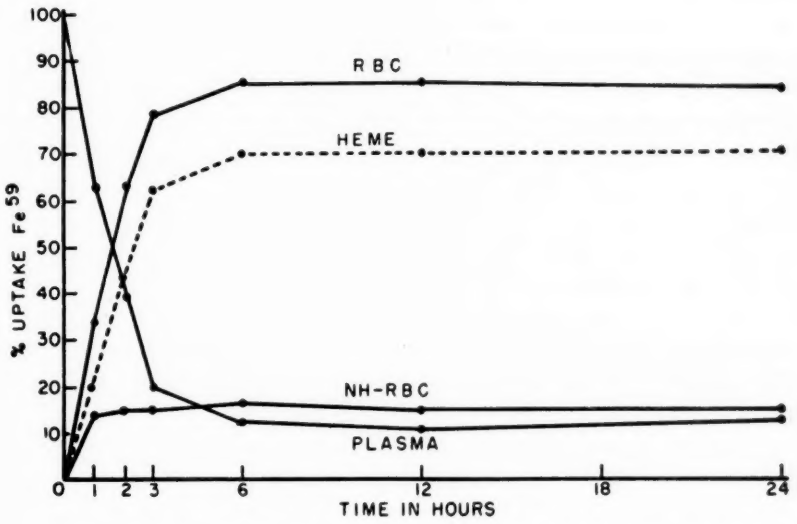

Fig. 1.—The disappearance of iron[59] from the plasma and its uptake into duck erythrocytes. NH-RBC refers to nonheme red blood cell iron. This represents the difference between the total RBC iron[59] and heme radioiron.

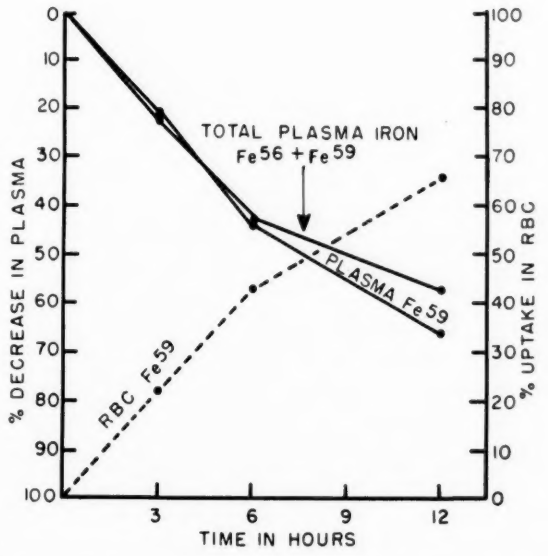

Fig. 2.—Comparison of the disappearance rate of iron from plasma as determined by chemical and isotopic methods. The results are expressed as per cent decrease from the initial concentration.

To determine if the radioiron added to the plasma is utilized at the same rate and to the same extent as the native plasma iron, the rate of disappearance of the iron from plasma was determined by both isotopic and chemical methods. To 10 ml. aliquots of blood (V.P.R.C.,* 45 ml./100 ml.; reticulocytes, 5 per cent; plasma iron concentration, 261 μg per cent) obtained from a single duck, 2 ml. of 0.15 M sodium chloride solution was added which contained 0.1 μg iron with an activity of 0.0082 μc iron^{59}. The total plasma iron and the iron^{59} were determined after incubation for 3, 6, and 12 hours at 37° C. As shown in Fig. 2, within the experimental error of the total plasma iron determination, the rate and degree of removal of iron from the plasma was the same by both methods. This would indicate that the radioiron represented a true tag of the plasma iron pool.

An experiment was designed to determine if iron, once it has been incorporated into heme, is in equilibrium with nonheme erythrocyte iron and the plasma iron. If such were the case, the measurement of heme iron^{59} at any given time would not be a measure of heme synthesis. A duck was injected intravenously with a relatively large amount of radioiron, and, four days later, after most of the radioiron had been incorporated into heme, blood was removed and incubated at 37° C. for 12 hours (Table I). During this period of time there was no decline in the radioactivity of the hemin fraction, nor was an increase noted in the nonheme or plasma compartments. This would seem to indicate that there is no exchange of heme iron into the other compartments.

TABLE I. THE FAILURE OF HEME IRON TO EXCHANGE WITH NONHEME AND PLASMA IRON DURING IN VITRO INCUBATION

TIME OF INCUBATION (HOURS)	% RADIOACTIVE IRON				
	WHOLE BLOOD	RBC	HEME	NH-RBC*	PLASMA
0	100	95	83	17	0.2
3	100	95	81	19	0.5
6	100	97	80	20	0.3
12	100	93	85	15	0.2

1.37×10^6 c.p.m. Fe^{59} were injected intravenously into a duck. Four days later the blood was removed and incubated at 37° C.

*Refers to nonheme RBC iron.

The reproducibility of the methods used for the determination of the total erythrocyte and hemin radioactivity is indicated in Table II. Triplicate determinations of percentage of uptake of iron^{59} into red cells were performed on three different specimens of blood after incubation for six hours at 37° C. Three or more determinations of the percentage of uptake of radioiron into heme were made on three different specimens of blood after six hours' incubation at 37° C. In all experiments the triplicates were run individually from the beginning and treated as individual determinations. Based on the 9 determinations of iron^{59} uptake in red cells and the 13 determinations of iron uptake in the heme, the standard deviation of an individual red cell iron^{59} determination was ± 2.5 per cent and of an individual heme determination was ± 3.5 per cent.

*Volume of packed red cells.

Factors Influencing the Uptake of Radioiron in Vitro by Duck Erythrocytes.—In order to determine the effect of the plasma iron concentration on the uptake of iron, aliquots of pooled erythrocytes were incubated for 12 hours in normal plasma to which increasing amounts of nonradioactive iron were added. To each flask was added 0.0082 μc of radioiron. The results are presented in Table III.

TABLE II. REPRODUCIBILITY OF THE METHODS

	% UPTAKE OF Fe^{59} INTO RBC EXPERIMENT			% UPTAKE OF Fe^{59} INTO HEME EXPERIMENT		
SAMPLE	1	2	3	4	5	6
1	48	62	70	79	70	38
2	50	62	67	75	70	38
3	47	65	72	74	72	43
4					70	43
5						32
Mean	48	63	70	76	70	39

TABLE III. THE EFFECT OF THE AMOUNT OF IRON IN THE PLASMA ON THE IRON UPTAKE BY DUCK ERYTHROCYTES

	Fe AVAILABLE (μG)	UPTAKE					
		RBC		HEME		NH-RBC*	
SAMPLE		(%)	(μG)	(%)	(μG)	(%)	(μG)
1	7.3	77	5.6	67	4.9	10	0.7
2	14.1	60	8.5	46	6.5	14	2.0
3	24.4	42	10.2	33	8.0	11	2.2
4	41.6	29	12.1	18	7.5	11	4.6
5	73.8	19	14.1	10	7.4	9	6.6
6	145.3	11	14.5	5	7.3	5	7.2

Each sample contained 8.5 ml. packed RBC, 8 per cent reticulocytes, and 11.5 ml. plasma. The amount of iron in the plasma was varied by the addition of a solution of ferric chloride. Cell suspensions were incubated 12 hours.

*Nonheme RBC iron.

With increasing amounts of nonradioactive iron, the percentage of the radioiron taken up by the erythrocytes decreased. When insufficiency of iron was the limiting factor (Samples 1 and 2, Table III), the amount of iron incorporated into heme increased with increasing amounts of iron. When more than 24 μg of iron were available, however, there was no further increase in the amount of iron incorporated into heme, but the iron in the nonheme red-cell fraction continued to increase.

To observe more clearly the influence of the quantity of plasma iron on the iron uptake by erythrocytes, increasing quantities of nonradioiron were added to four 20 ml. aliquots of pooled duck blood (V.P.R.C., 50 ml./100 ml.; reticulocytes, 19 per cent) to give the total plasma iron concentrations shown in Fig. 3. Radioiron (0.0082 μc) was added to each sample and the flasks were incubated at 37° C. for 24 hours. At 3, 6, 12, and 24 hours, samples were removed for the determination of the red-cell radioactivity. In the lower portion of Fig. 3 it may be seen that the lower the plasma iron concentration, the greater and more rapid was the proportion of radioiron taken up. From

the upper portion of Fig. 3 it is clear that the rate of uptake of iron is constant. When lack of iron is not the limiting factor, the rate is independent of the plasma iron concentration.

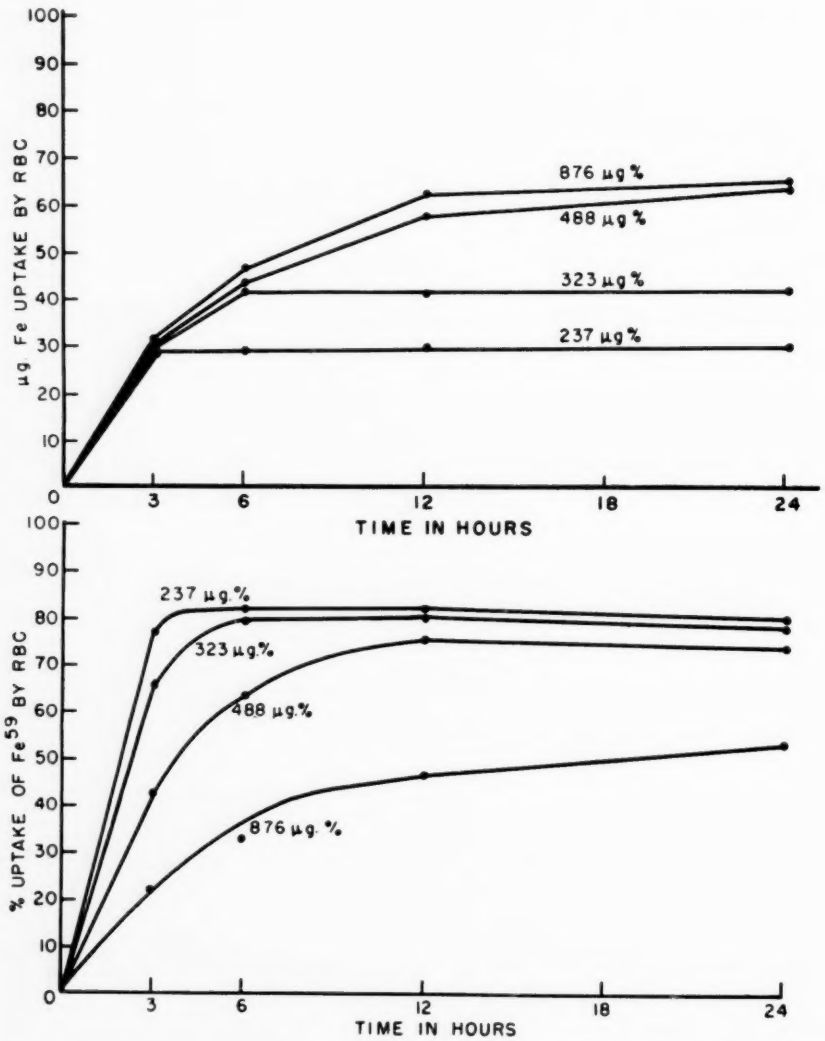

Fig. 3.—The relation of the plasma iron concentration to the per cent of iron[59] (bottom) and the absolute amount of iron (top) taken up by duck erythrocytes. The numbers on the lines refer to the plasma iron concentration in µg/100 ml.

To compare the ability of immature circulating red cells with that of more mature ones to take up iron, the influence of the degree of reticulocytosis was studied. Approximately 30 per cent of the total blood volume of a duck was removed on day 0. On an aliquot of this blood, red-cell iron uptake was determined in vitro. In order to avoid variations which might be related to

different plasmas, plasma from the remainder of the blood samples was stored and used later for suspension of the cells removed on subsequent experimental days. To obtain specimens of blood with various quantities of reticulocytes, 20 ml. of blood were removed on days 2, 4, 6, 8, and 15. The cells were separated, washed three times in saline, and 2.6 ml. packed cells were resuspended in 3.0 ml. of the plasma obtained on day 0. Ferric chloride was added to the plasma to provide a total of 24 μg of iron.

In Fig. 4 the iron uptake, expressed in micrograms/2.6 ml. of packed cells, is plotted, along with the percentage of reticulocytes in the blood. As may be seen, there was an excellent correlation between the amount of iron uptake and the number of reticulocytes present.

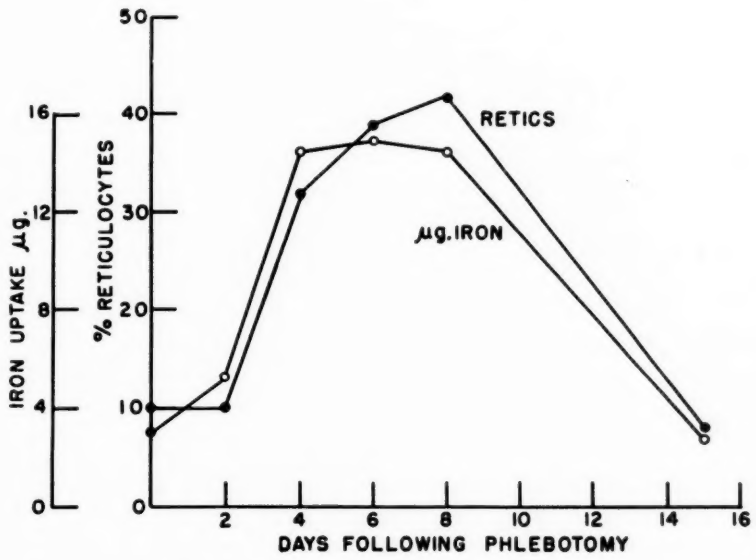

Fig. 4.—The correlation between the iron uptake in micrograms and the per cent reticulocytes in the sample. (For details consult text.)

The inhibiting influence of several factors on this system was also studied. To determine the effect of temperature, samples of blood were incubated at 37° and 5° C. (Table IV). At a temperature of 5° C. there was much less uptake of iron into heme than at 37° C. There was less difference in the nonheme iron. Potassium cyanide in 1×10^{-2} M concentration abolished the uptake of iron into heme, but failed to influence the nonheme iron fraction. A combination of potassium cyanide and incubation at 30° or 10° C. failed to alter nonheme iron uptake.

Desoxypyridoxine, a pyridoxine antagonist, in an amount greater than 1.5 mg. per 11.6 ml. (0.5×10^{-2} M) produced a marked suppression of iron uptake into heme but again failed to modify the uptake of iron into the nonheme fraction (Table V). The simultaneous addition of pyridoxine hydrochloride and

TABLE IV. THE INFLUENCE OF TEMPERATURE AND OF POTASSIUM CYANIDE ON IRON UPTAKE BY DUCK ERYTHROCYTES

EXPERIMENT	TEMPERATURE (° C.)	KCN 1×10^{-2} M	IRON UPTAKE (μG) RBC	HEME	NH-RBC*
I	37		37.0	32.0	5.0
	5		4.7	1.5	3.2
II	37		15.4	8.2	7.2
	37	+	8.7	0.0	8.7
	30	-	9.1	0.0	9.1
	10	-	8.5	0.0	8.6

Each sample contained 10 ml. packed RBC, 10 per cent reticulocytes, and 12 ml. plasma. There were 64 μg iron present in each sample in Experiment I and 25 μg iron present in each sample in Experiment II.

*Nonheme RBC iron.

desoxypyridoxine in ratios of 1:1 to 5:1 did not prevent the inhibiting effect of desoxypyridoxine (Fig. 5). The addition of the vitamin to the cell suspension 30 minutes prior to the addition of the antagonist also failed to prevent the inhibitory effect of the desoxypyridoxine. A folic acid antagonist, 4-aminopteroylglutamic acid, in a concentration of 5×10^{-3} M, or the addition of pantoyltaurine, a pantothenic acid antagonist, in a concentration of 2×10^{-5} M, had no influence on the uptake of iron by erythrocytes.

TABLE V. THE EFFECT OF DESOXYPYRIDOXINE ON IRON UPTAKE BY DUCK ERYTHROCYTES

SAMPLE	DB6 (MG.)*	IRON UPTAKE (μG) RBC	HEME	NH-RBC†
1	0.0	6.7	6.0	0.7
2	0.5	6.7	5.7	2.0
3	1.0	6.6	5.6	2.0
4	1.5	6.5	5.5	2.0
5	3.0	5.5	4.2	1.3
6	6.0	2.9	1.4	1.5

Each sample contained 8.4 ml. RBC, 2 per cent reticulocytes and 11.6 ml. of plasma. There were 7.3 μg iron in the plasma. The cell suspensions were incubated for six hours.

*DB6 refers to desoxypyridoxine hydrochloride.

†Nonheme RBC iron.

To ascertain whether or not there are factors in plasma other than iron which favor the uptake of iron into erythrocytes and hemin, 70.4 ml. of three-times-washed, packed erythrocytes (reticulocytes, 12 per cent) were divided into eight aliquots of 8.8 ml. of packed cells each. To four flasks 0.15 M NaCl was added and, to the other four, a similar volume of normal duck plasma was added. A solution of ferric chloride was added to the flasks containing saline so that the amount of iron in all the flasks was equivalent to 26 μg. After incubation at 37° C. for 1, 3, 6, and 12 hours, the cells were centrifuged free of plasma, washed three times with cold isotonic saline, and measurements were made of the activity of the radioiron in the erythrocytes. The cells were then hemolyzed and the hemin was isolated. The results are presented in Fig. 6. The uptake of iron by the cells suspended in 0.15 M sodium chloride was approximately 35 per cent less than that of the cells suspended in plasma. The decrease in the iron uptake by the cells in the saline solution was accountable for by a

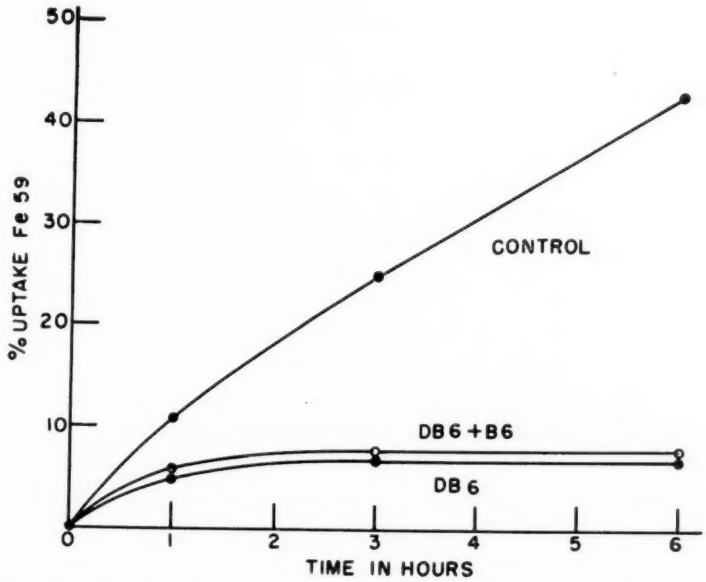

Fig. 5.—The influence of desoxypyridoxine (DB6) on the uptake of iron[59] by duck erthrocytes. The addition of pyridoxine (B6) to the desoxypyridoxine in a ratio of 1:5 did not prevent the inhibiting effect of the desoxypyridoxine. (For details consult text.)

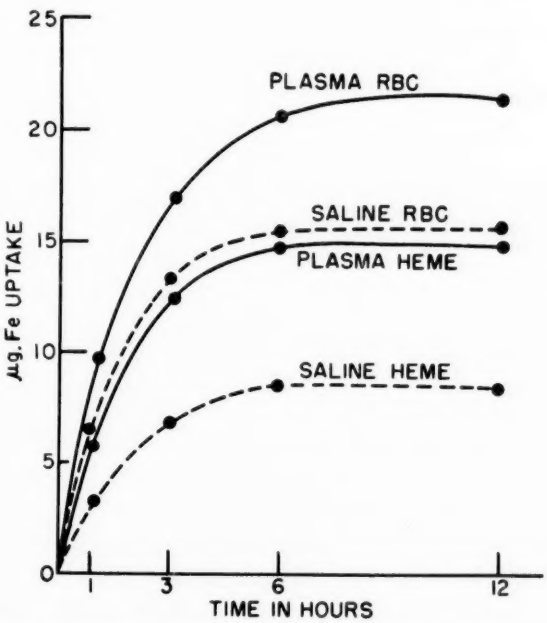

Fig. 6.—The uptake of iron into erythrocytes and heme by cells suspended in 0.15 M sodium chloride as compared with normal duck plasma. (For details consult text.)

reduction in the heme fraction, the nonheme fraction being equal in the cells suspended in the two different media. The addition of vitamin B_{12}, cobalt, glycine, folic acid, cortisone, thyroxine, or adrenocorticotrophic hormone failed to increase the synthesis of heme when the cells were suspended in either saline or plasma.

Duck erythrocytes were incubated with plasma obtained from ducks previously phlebotomized and with plasma from ducks with polycythemia produced by transfusion. The results were compared with the values obtained when similar cells were incubated with plasma removed from normal ducks. From experiment to experiment the data were extremely inconsistent and no conclusions could be drawn. For these reasons the data are not presented.

Comparison of the Uptake in Vitro of Radioiron by Human and Duck Erythrocytes.—A comparison between the uptake of iron by human and duck erythrocytes is presented in Table VI. Cells from normal human subjects took up negligible quantities of iron, whereas in the blood from a patient with hemolytic anemia where there were 33 per cent reticulocytes, appreciable quantities of iron were taken up. The iron uptake of human reticulocytes, however, was only about one-fifth of that of duck blood containing a similar number of reticulocytes. It is of course to be noted that all of the red cells of ducks are nucleated, including the reticulocytes. It may be added that human fetal erythrocytes, as obtained from umbilical blood, took up more iron than did adult cells. Whether or not this was related to the presence of fetal hemoglobin in the newborn red corpuscles is unknown.

TABLE VI. THE UPTAKE OF IRON INTO HUMAN RED BLOOD CELLS AS COMPARED WITH AVIAN CELLS

SAMPLE	RETICULOCYTES (%)	IRON UPTAKE (μG)
Normal human subject	0.8	0.12
Normal human subject	0.3	0.17
Normal human subject	0.8	0.07
Hemolytic anemia*	33.0	5.50
Duck blood*	1.0	4.10
Duck blood*	32.0	25.70
Umbilical vein (human)	2.6	0.72†
Umbilical vein (human)	2.0	1.14

The 10 ml. of whole blood were incubated at 37° C. for six hours. There were 4.6 ml. packed red cells, 5.4 ml. plasma, and 7.9 μg iron in each sample.

*The plasma contained 46 μg of iron.

†Uptake into heme rather than total red cell.

DISCUSSION

From the data presented it is evident that duck erythrocytes incubated at 37° C. take up iron when suspended in plasma or 0.15 M sodium chloride. This confirms previous studies.[10] In such a system approximately 85 per cent of the iron which enters the erythrocytes becomes irreversibly incorporated into heme and 15 per cent is held within the cell in a form which has been termed the "nonheme RBC iron pool."

The quantity of iron taken up by a given number of avian nucleated red cells depends upon the amount of iron available in the plasma, the temperature at which the incubation is carried out, and the proportion of cells which contain reticulum. When lack of iron is not the limiting factor and the incubation is carried out for six or more hours at a temperature of 37° C. and pH of 7.4, the amount of iron taken up is related to the number of reticulocytes present. Mature nonreticulated cells are able to synthesize heme only to a limited extent. This is in agreement with the observations of Allfrey and Mirsky.[7]

The capacity of duck erythrocytes to synthesize heme is indicated by our data, from which it can be calculated that in 100 ml. of normal duck blood with a volume of packed red cells of 42 ml./100 ml., 6 per cent reticulocytes, and a plasma iron concentration of 180 μg per cent, approximately 396 μg of heme can be synthesized. To synthesize this quantity of heme, approximately 36 μg of iron and 360 μg of protoporphyrin would be required. If the newly synthesized heme is completely converted into hemoglobin, approximately 11 mg. of new hemoglobin would be produced. Since the amount of hemoglobin initially present was 13.70 Gm., this would represent an increase in hemoglobin of approximately 0.08 per cent. Such an increase would have a negligible influence on the mean corpuscular hemoglobin concentration. For example, this would increase the mean corpuscular hemoglobin concentration from 32.62 to 32.65 per cent.

A sample of blood similar to the one just cited but containing 32 per cent reticulocytes rather than 6 per cent, and with a plasma iron concentration greater than 350 μg per cent, would be capable of synthesizing 2.23 mg. of heme or 61 mg. of new hemoglobin. This would require 203 μg of iron and 2,040 μg of protoporphyrin and would represent an increase in the hemoglobin of 0.45 per cent. Again there would be an insignificant increase in the mean corpuscular hemoglobin concentration.

The above calculations of 0.08 to 0.45 per cent synthesis of hemoglobin compare favorably with the value of 0.4 per cent obtained from the calculation of heme synthesis by the N^{15} glycine method.[2]

Since normal duck erythrocytes contain only about 60 to 100 μg of free protoporphyrin per 100 ml. of packed cells* and 360 to 2,040 μg were required, it follows that new porphyrin synthesis must also have occurred in these experiments.

Since the method used measures heme synthesis and not hemoglobin synthesis, the above calculations for hemoglobin synthesis are based on the assumption that all of the newly formed heme was converted into hemoglobin. That this assumption is reasonably valid is indicated by the observation of Neuberger[6] that globin synthesis roughly parallels the rate of porphyrin synthesis and the observation of Thorell[15] that the formation of protein in the developing cell precedes that of porphyrin formation.

The demonstration of a nonheme red-cell iron fraction is not a new observation. Discrepancies between the iron content and the oxyhemoglobin

*Unpublished observations by Mr. Alex Krammer in this laboratory.

content of avian blood as determined by the manometric apparatus have been reported by several investigators.[16, 17] The size of this fraction as determined by various workers ranges from 1.6 per cent[17] to 12.7 per cent[18] of the total red cell iron. The results of our experiments suggest that the amount of nonheme iron present in the cell depends upon the plasma iron concentration (Table III). Furthermore, when the amount of iron present in the suspending medium was insufficient for optimal heme synthesis, less NH-RBC iron was produced. The observation that the NH-RBC iron was little or not at all influenced by potassium cyanide or by reduction in the temperature suggests that the iron diffuses into the cell by a nonenzymatic process.

The observation that heme synthesis is considerably less when cells are suspended in 0.15 M sodium chloride than when suspended in normal plasma suggests that the system we have employed may be of value in a study of the influence of various added substances on hemoglobin synthesis. There are, however, a number of limitations. Since the cells are in the phase of declining differentiation,[15] only the factors which influence this phase can be studied. The role of the permeability of the erythrocyte membrane to a given substance is always a problem, as pointed out by London and Yamasaki.[19] The failure of a substance to influence the system may simply mean that it is incapable of crossing the membrane. The system does not measure porphyrin synthesis per se or the incorporation of iron into protoporphyrin but rather over-all heme synthesis. Duck erythrocytes are normally supplied with a reserve of hemoglobin precursor substances including iron, glycine,[20] and preformed porphyrins. Consequently little additional material other than iron is needed. It may be that the synthetic capacities of these cells are limited by factors other than a supply of hemoglobin precursor substances. Finally, the state of the iron in the suspending media is an important factor. When ferric chloride is added to plasma, as shown in these experiments, it is handled in the same fashion as the native plasma iron. However, if salts which are capable of forming insoluble iron compounds are present in the medium or if substances are present which are capable of complexing with iron, then the iron kinetics will be greatly altered. To date, all of our attempts to study iron uptake from complex synthetic media have been unsuccessful.

It is interesting that iron added to plasma in the form of ferric chloride is handled in the same fashion as the native plasma iron. In other species, such as man[21] and the pig,[22] the iron in the plasma is bound to transferrin, the β_1 globulin metal-binding protein. In ducks we have been unable to demonstrate* any capacity of the plasma to bind iron by the usual technique.[23, 24] Furthermore, when iron bound to human transferrin is added to duck plasma, it is removed much more slowly than the native plasma iron.* This is perhaps not surprising since the transferrin is a protein from another species. Whether or not the duck has the same type of iron transport system as the human subject remains to be demonstrated.

*Unpublished observations.

SUMMARY

Duck erythrocytes suspended in normal duck plasma incorporate radioiron into the erythrocytes. The rate of incorporation of iron is most rapid in the first six hours and is complete in less than 24 hours. The amount of iron incorporated is maximal at 37° C. and depends upon the plasma iron concentration and the percentage of reticulocytes present. Eighty to ninety per cent of the iron taken into the cells is incorporated into heme. Iron, once incorporated into heme, does not exchange with the plasma. Ten to twenty per cent of the iron taken into the erythrocytes is in a nonheme state. The size of this fraction depends upon the plasma iron concentration. Potassium cyanide, desoxypyridoxine, and low temperature inhibit heme synthesis but have little or no influence on the amount of nonheme iron.

Duck erythrocytes suspended in 0.15 M sodium chloride incorporate less iron into heme than when suspended in plasma.

Human reticulocytes take up about one-fifth the amount of iron as compared to a similar quantity of duck nucleated reticulocytes. Human fetal cells take up more iron than do normal adult cells.

We are indebted to Miss Betty Tatting, Miss Doris Kurth, Mr. George Trappett, and Mr. Ocie Hadley for technical assistance. The desoxypyridoxine, pyridoxine, pyridoxal, cortisone, and vitamin B_{12} were furnished by Dr. Karl Folkers, Merck & Company, Rahway, N. J. Dr. Thomas Jukes, Lederle Laboratories, Pearl River, New York, supplied the 4-aminopteroylglutamic acid and the pteroylglutamic acid.

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INTERFERENCE WITH FIBRIN FORMATION IN MULTIPLE MYELOMA BY AN UNUSUAL PROTEIN FOUND IN BLOOD AND URINE

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THE disturbances in plasma proteins associated with most cases of multiple myeloma are of great clinical and experimental interest, particularly in view of their probable relation to metabolism by malignant tissue.[1-3] These alterations in the pattern of plasma proteins are both quantitative and qualitative, as in the altered physiochemical properties of cryoglobulins and Bence-Jones protein. The appearance of abnormal peaks in the electrophoretic analysis is an almost uniform finding.[4, 5] The clinical derangements, sometimes attributed to altered protein synthesis, include pathologic renal changes, alterations in the colloidal stability of the plasma and, more rarely, disturbances in hemostasis.

Recently, cases in which a hemorrhagic diathesis was attributed to impaired fibrinogen conversion have been reported from European clinics. The localization of the clotting defect to interference with fibrinogen conversion by the protein disturbance was considered clearly established in the two cases reported by Luescher and Labhart[6] and Uehlinger.[7] These authors concluded that the conversion of fibrinogen was blocked at an intermediary phase ("profibrin"[8-11]) by the abnormal protein complex acting as a protective colloid.

These investigators emphasized that hyperproteinemia, or hyperglobulinemia, per se does not necessarily embarrass blood clotting, a concept which might be inferred from the writings of Waldenstrom,[12-14] who has studied several cases of purpura with concomitant abnormalities of the globulins of the blood. It is far from clear what mechanism underlies the impaired hemostasis in all these cases of "purpura hyperglobulinemica" and "purpura macroglobulinemica."[12-14] Some have reported circulating anticoagulants in such diseases as lupus erythematosus, leukemia, etc.,[15] where alterations in the pattern of plasma proteins are found. Others have found only quantitative or qualitative platelet defects. The conclusion that the hyperglobulinemia is the cause of the hemorrhagic tendency is not always justified. In those cases described by Uehlinger[7] and Luescher and Labhart,[6] however, the locus of coagulation disturbance was well demarcated and proved to be related to the abnormal globulins.

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This investigation was supported in part by a research grant from the Cancer Research Coordinating Committee of the University of California and The American Cancer Society.

Received for publication, June 9, 1953.

*John and Mary Markle Foundation Scholar in Medical Science.

The purpose of this paper is to report a similar case, although no hemorrhagic manifestations were present. Data are presented to clarify further the manner in which the abnormal protein affects fibrin formation and how this seems to be partly related to the calcium-binding property of the abnormal protein. Furthermore, the remarkable finding is presented that the urinary protein, and that in the plasma, exhibited similarities both as to physical properties and biologic action.

CASE REPORT

Case F. L. (G.H. No. 35061) was admitted Sept. 4, 1952. This 66-year-old white married engineer was in good health up until one year prior to admission when he developed severe lower back pain following a fall. He received symptomatic therapy, but derived no relief. X-ray of the spine revealed multiple vertebral fractures, and a presumptive diagnosis of multiple myeloma was made.

Six months prior to admission he developed pain and stiffness in the region of the cervical spine. Study of the bone marrow revealed numerous plasma cells and the clinical impression of myeloma was confirmed. Two months before admission, he was treated with a course of nitrogen mustard following which he became severely debilitated. He was admitted to this hospital because of weakness and a 20 pound weight loss. The past history and system review were noncontributory.

The positive findings on physical examination were confined to the skeletal abnormalities. The patient walked in a stooped position. He held his head and back rigidly, the slightest motion producing pain. There was marked spasm of the cervical and back muscles. A severe kyphosis in the thoracic region was noted.

Laboratory Data.—In addition to the electrophoretic and clotting studies covered in detail later, the following routine data are of interest: red blood count 2.87 million, hemoglobin 11.6 Gm. per cent, volume of packed red blood cells 34 per cent, marked rouleaux formation on blood smear, white blood count 5,000 per cubic millimeter with a normal differential cell count. Bence-Jones protein was found on urine examination (heat and acetic acid test, Osgood-Haskins test, and sulfosalicylic acid test); the urine was otherwise normal. Total plasma protein 10.4 Gm. per cent with albumin 3.0 Gm. per cent and globulin 7.4 Gm. per cent (ratio 0.45), calcium 10.0 mg. per cent, phosphorus 3.6 mg. per cent, alkaline phosphatase 2.6 Bodansky units, urea nitrogen 12.0 mg. per cent, creatinine 1.0 mg. per cent. On subsequent examinations the serum calcium was 9.7 mg. per cent and 9.5 mg. per cent.

X-rays revealed marked demineralization of the lumbar spine and pelvis, and osteolytic, punched-out lesions in the skull, femora, ribs, and scapulas.

Bone marrow examination showed the marrow to be largely composed of plasma cells of the myeloma type.

Course in Hospital.—The patient's condition remained fairly stable without improvement in symptoms for about two months. He was treated symptomatically with blood transfusions as required, and a lumbosacral corset. He was discharged home, but soon his condition deteriorated, requiring readmission to the hospital. He became progressively weaker and died on Feb. 15, 1953, four and one-half months after initial admission. Post-mortem examination confirmed the clinical diagnosis of multiple myeloma.

METHOD OF ELECTROPHORESIS

Five ml. of *plasma* was diluted with 10 ml. of Veronal buffer and dialyzed against the buffer for 16 hours at 2° C. Electrophoresis was carried out in the tall form of the standard 11 ml. Tiselius cell at a field strength of 6.3 volts per centimeter at a water bath temperature of 2° C. The current was passed for 3 hours in this case, since it was found that the

fibrinogen peak was not resolved from the large abnormal peak at the end of two hours. The patterns were photographed by the Schlieren method, and the areas of the peaks were obtained from photographic enlargements by the use of a planimeter.

Urinary Proteins.—Twenty-four hour collections were made and kept refrigerated. A 500 ml. aliquot was shell frozen and lyophilized. The lyophil was reconstituted in 50 ml. of distilled water and 15 ml. of this was dialyzed against 375 ml. of the Veronal buffer which was changed at three successive regular intervals over the course of 84 hours of dialysis. Henceforth, the urinary protein was handled as designated above for the plasma proteins with the exception of the duration of the passage of the current which, for the urinary proteins, was two hours since very little migration occurred after this period of time.

Electrophoretic Results.—The ascending and descending patterns for ten samples of plasma and three samples of urine were obtained in this case. Since they all showed relatively the same abnormalities, only the descending patterns obtained on Sept. 23, 1952, are shown in Fig. 1.

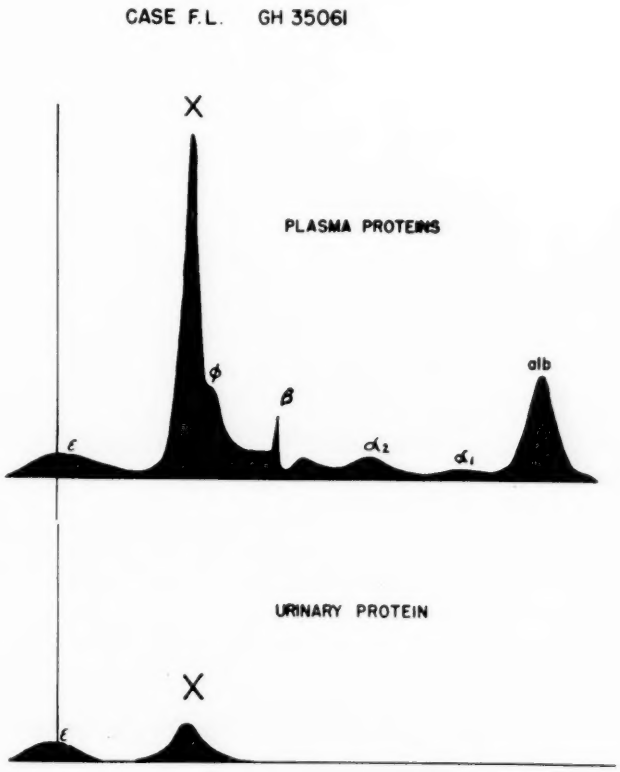

Fig. 1.—Showing the descending electrophoretic patterns of the plasma and urinary proteins. Note the sharp abnormal peak with mobility slightly slower than that of fibrinogen (pattern taken after three hours of migration), and the single homogeneous urinary protein (pattern taken after two hours of migration).

It will be noted that a large abnormal peak is present in the plasma and migrates with a mobility intermediate between that of fibrinogen and gamma globulin. The urine contained a slow-moving component which appeared to be homogeneous and relatively pure. The average concentrations and mobilities of the descending patterns are given in Table I.

HEMOSTATIC STUDIES

The abnormality of blood clotting was first noted when blood drawn for various chemical studies on the serum failed to show clot retraction with expression of serum.* Prolonged centrifugation failed to yield serum and no retraction was apparent after several days at room temperature.

TABLE I. PROTEIN CONCENTRATIONS DETERMINED BY ELECTROPHORESIS IN BLOOD AND URINE OF CASE F. L.

	CONCENTRATION IN GM. %	MOBILITY × 10^{-5}	CM.^{2} / SEC. VOLT
Total plasma proteins =	10.0		
Albumin =	2.6	5.1	
Total globulins =	7.4		
α 1 =	0.4	4.2	
α 2 =	0.8	3.3	
β =	0.9		
γ =	0.4		
"X" =	4.9	1.3	
A/G =	0.35		
Urinary protein =	0.24	2.12	

1. Bleeding time, 3 to 5 minutes.
2. Rumpel-Leede test, negative.
3. Clotting time, 6 to 8 minutes (Lee-White).
4. Clot retraction, absent.
5. Prothrombin activity, per cent normal, 100.
6. Accelerator globulin, per cent normal, 100.
7. Serum prothrombin conversion accelerator, normal.
8. Prothrombin consumption (Quick), normal.
9. Fibrinogen concentration (electrophoresis), normal.
10. Platelet count, 300,000/mm.^{3} (and normal in appearance on blood smear).
11. Tests for qualitative platelet defect, clot retraction not improved by addition of normal platelets to patient's blood. Patient's platelets behaved normally in platelet-poor normal blood, enhancing clot retraction. Prothrombin consumption studies using platelet-free normal plasma and comparing normal platelets with the patient's platelets gave equivocal results because of the difficulty in obtaining a platelet-rich suspension from the patient's blood.
12. Plasma clot by thrombin, an amount of thrombin which would consistently clot 1 ml. of normal plasma in 15 seconds failed to clot 1 ml. of the patient's plasma in less than 60 seconds.
13. Antithrombic activity of blood, using a modification of the method of Astrup,[16] no abnormality of antithrombic activity of the patient's blood could be found.
14. Action of fibrinolysin, activation of fibrinolysin by streptokinase produced similar lysis of the patient's and normal clots.

CHARACTERISTICS OF THE ABNORMAL CLOT AND SPECIAL STUDIES

As described, the blood congealed "en masse" and failed to express serum. Fig. 2 shows the large, clear clot (with the red cells layered at the bottom due to rapid sedimentation) and the lack of clot retraction.

*Attention was first called to this by Mrs. Francis Davis, Senior Laboratory Technician, Metabolic Research Laboratory, Wadsworth General Hospital.

In contradistinction to the unretracted clot of platelet deficient blood, this clot showed a firm, rubbery quality with no tendency to friability. The plasma clot which appeared after addition of thrombin was entirely clear without any of the opacity typical of fibrin formation. Hanging drop preparations observed microscopically showed no fibrin-strand development. Electron microscopic and photometric studies by Luescher and Labhart[6] and Uehlinger[7] previously indicated the absence of fibrin formation in their cases. It was noted that on addition of the thrombin, the plasma became gradually more oily and viscous until totally congealed, rather than exhibiting the latent period with terminal rapid fibrin formation as seen with normal plasma.

Dilution of the plasma, 1 to 10, with saline before clotting, yielded a different-appearing clot in which some fibrin seemed to form. This dilution phenomenon had been noted previously in the two cases described.[6, 7]

Admixture of the patient's plasma with normal plasma produced the same peculiar gel when thrombin was added. The addition of human fibrinogen to the patient's plasma also failed to produce a normal fibrin clot. This observation indicated the patient's fibrinogen was not structurally altered, but that the presence of the abnormal protein prevented fibrin formation in the ordinary manner.

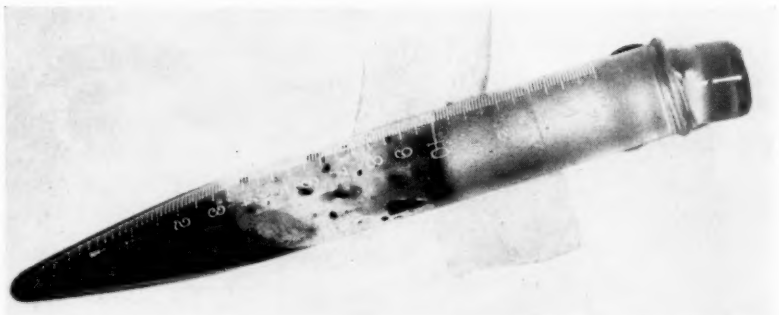

Fig. 2.—Photograph of blood clot as it formed spontaneously in the tube. No retraction after several hours. Note the translucency of the plasma clot which has been pulled up from the mass of sedimented red cells to show its solidity.

The delayed clot formation of the plasma on addition of thrombin was not due to antithrombin in the usual sense. This delayed response to thrombin was noted even more strikingly in the two previous cases cited. Further, it was observed in our case that the gel could be forcibly dispersed by stirring with a glass rod, most of the gel apparently going back into solution. The addition of more thrombin again resulted in gel formation. This process of gel formation with thrombin and forcible dissolution by stirring could be repeated many times. Eventually, however, a point was reached where no further gel would form on addition of thrombin. At no time during such manipulation was fibrin observed.

At this point, recalcification of the plasma produced immediate clotting, probably because of the large amount of thrombin present. The clot formed

had the characteristics of a fibrin clot. This surprising observation was repeated several times.

Calcium ions have been shown to influence the fibrinogen → fibrin transformation. Thus, a "fibrinoplastic" or accelerating property, by no means specific to calcium, was shown by Smith and associates.[17] Robbins[18] demonstrated certain differences in the characteristics of the fibrin formed, depending upon the presence of calcium ions in the clotting mixture. Several investigators have demonstrated that calcium increases tensile strength of the fibrin clot. More recently, Laki and Lorand[19] made the observation that fibrin clots formed from purified fibrinogen by thrombin are soluble in urea solution. Further study by Lorand[20] indicated that calcium ions and a factor present in serum are both required to give clots which are insoluble in 30 per cent urea. Loewy,[21] using agents which block sulfhydryl groups, found that if the presumed disulfide linkages were prevented, the clots which formed were soluble in 30 per cent urea, suggesting that two types of molecular linkages probably occur during clotting of fibrinogen. This function of calcium in regard to the nature of the fibrin clot has been recently confirmed by Ferry and associates[22] using quantitative methods. The presence of calcium and a serum factor seem necessary, therefore, for the formation of at least one type of fibrin network.

In view of these reports, and the observation that ionic calcium seemed necessary for the formation of a fibrin network in this patient's blood, further studies on the relationship of the calcium ion concentration were carried out. Table II indicates the effect of varying concentrations of ionized calcium on the speed of clotting by thrombin.

TABLE II. THE EFFECT OF VARYING CONCENTRATIONS OF CALCIUM ON THE SPEED OF CLOTTING OF THE PATIENT'S PLASMA BY THROMBIN

	1	2	3	4	5	6	7	8
Ml. patient's plasma (0.1 M oxalate ⅕ blood)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Calcium chloride in buffer (0.5 ml.), molarity		1.0	0.5	0.25	0.12	0.06	0.03	0.015
Ml. imidazole buffer saline, pH 7.2	1.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Units thrombin (100 u./ml.)	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Clotting time (sec.)	52.5	α	α	58.0	11.5	18.6	60.0	60.0

It was found that clotting was most rapid at a concentration of calcium some six or seven times that necessary to replace the calcium bound by oxalate. It is notable that, even at this concentration, clotting was slower than normal with the amount of thrombin used. The appearance of the clots at different calcium concentrations indicated definite fibrin formation in those tubes with excess calcium. The appearance was that of a gel shot through with opaque fibrin strands. The fibrin could be separated from the gel with a stick.

Table III illustrates the deleterious effect of the patient's plasma, serum, and urinary protein on the quality of clot formed when these are mixed with

normal plasma and the corrective influence of ionized calcium. This ability of ionic calcium in excess to improve the quality of the clot and alter its solubility in urea solution is well shown by comparing tubes 4 and 5. It is apparent that the clots from mixtures of normal plasma and the patient's plasma were more soluble in urea, in the absence of ionized calcium, than the patient's clot alone, although both were improved by calcium. No explanation for this is readily apparent. The effect of calcium on the urea solubility of normal fibrinogen clotted with thrombin is seen in tube 9, as compared with tube 8. This confirms the observations of Laki and Lorand.[19] Normal serum alone did not affect the gel formation in the patient's plasma (tube 6), presumably because the amount of ionized calcium was not sufficient. The patient's serum exerted a deleterious effect on the clotting of normal plasma (tube 10), an effect which was corrected by addition of excess calcium.

Perhaps the most striking feature of this protocol is the effect of the urinary protein on the clot of normal plasma. As shown by tube 12, a very imperfect clot formed which was rapidly dissolved in urea. Calcium completely altered this effect and the resultant clot in the same system with calcium was typical fibrin, insoluble in urea.

These data were repeated several times and results were similar whether 30 or 60 per cent urea was used.

Table IV summarizes a typical protocol using bovine fibrinogen and demonstrates the same effect of calcium in improving the final clot when added to systems containing fibrinogen and the patient's plasma, serum, or urinary protein. It was noted that the deleterious effect of the urinary protein on the clotting of bovine fibrinogen was only slightly modified by calcium alone, whereas the presence of normal serum and calcium in the system enabled definite fibrin formation, the clot being insoluble in urea. These observations concur with those of Laki and Lorand,[19, 20] who showed that calcium and a normal plasma or serum factor are necessary for final fibrin formation, insoluble in urea.

The urinary protein had no inherent clotting ability. Thus, when added to systems containing thrombin or the components leading to thrombin evolution (i.e., serum containing prothrombin, calcium ions, human brain thromboplastin, accelerator-globulin, and serum prothrombin conversion accelerator) no clot or gel formed. However, if fibrinogen was also in this "complete" system, imperfect clotting occurred, which could be improved by an excess of calcium. The effect of the urinary protein on the physical properties of the fibrin clot leaves little doubt as to the basic similarity of the urinary protein with that in the plasma. Efforts to isolate the abnormal protein from the plasma by salt fractionation were unsuccessful, since fibrinogen and the abnormal protein exhibited such similar properties. Apparently, the patient's kidneys achieved separation of the two by virtue of the difference in molecular size or configuration.

TABLE III. THE EFFECT OF CALCIUM ON THE FINAL CLOT AS INFLUENCED BY THE PATIENT'S PLASMA, SERUM, AND URINARY PROTEIN, AS COMPARED WITH NORMAL PLASMA

	1	2	3	4	5	6	7	8	9	10	11	12	13
Ml. patient's plasma (0.1 M oxalate 1/9 blood)	0.5	0.5	0.5	0.3	0.3	0.3	0.3						
Ml. patient's urinary protein*												0.5	0.5
Ml. normal plasma (oxalated, fresh)				0.2	0.2			0.5	0.5	0.3	0.3	0.5	0.5
Ml. normal serum†						0.2							
Ml. patient's serum‡							0.2			0.2	0.2		
Ml. calcium chloride, 0.02 M (in buffer)		0.5							0.5				
Ml. calcium chloride 0.1 M (in buffer)			0.5		0.5						0.5		0.5
Ml. imidazole buffered saline, pH 7.2	0.5			0.5		0.5	0.5	0.5		0.5		0.5	
Units thrombin (100 u./ml.)	20.0	20.0	20.0	20.0	20.0	10.0	10.0	20.0	20.0	10.0	10.0	20.0	20.0
Appearance of clot	CG	GF	F+	CG	F++	CG	GF	F++	F++	F±	F+	GF	F++
Urea sol. 30%§ remaining clot													
2 hours	CG	GF	F+	(−)	F++	(−)	(−)	F+	F++	F±	F+	(−)	F++
24 hours	(−)	GF	(−)	(−)	F++	(−)	(−)	±	F++	(−)	F+	(−)	F++

Key: CG, Clear gel (no fibrin).
GF, Clear gel with some visible fibrin strands.
F±, Small, delicate fibrin clot.
(−), Complete disappearance of clot or gel.
F+ and F++, Definite opaque fibrin clot (no gel).

*Reconstituted in imidazole buffered saline, pH 7.2 at 5 Gm. per 100 ml.
†Diluted with equal parts buffered saline.
‡Obtained from plasma by clotting with thrombin and diluting with equal parts of 0.02 M $CaCl_2$ in buffer.
§Volume of urea solution equal to volume of system. Results duplicated with 60 per cent urea solution.

TABLE IV. THE EFFECT OF CALCIUM ON THE CLOTTING OF BOVINE FIBRINOGEN BY THROMBIN AS ALTERED BY THE PATIENT'S PLASMA, SERUM, AND URINARY PROTEIN

	1	2	3	4	5	6	7	8	9
Ml. fibrinogen sol. (500 mg./100 ml.)*	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ml. patient's plasma	0.5	0.5							
Ml. patient's urinary protein						0.5	0.5	0.5	
Ml. patient's serum			0.5	0.5					
Ml. normal serum					0.5			0.3	
Ml. $CaCl_2$, 0.02 M in buffer									0.5
Ml. $CaCl_2$, 0.1 M in buffer		0.5		0.5			0.5	0.2	
Ml. imidazole buffered saline, pH 7.2	0.5		0.5		0.5	0.5			
Units thrombin (100 u./ml.)·	20.0	20.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Appearance of clot	GF	F++	GF	F++	F+	CG	CG	F+	F++
Urea sol. 30%† clot remaining									
2 hours	GF	F++	GF	F++	F+	(−)	±	F+	F+
24 hours	CG	F++	CG	F++	F+	(−)	(−)	F±	F±

Key: As in Table III.

*Armour Fraction I (recalcified with sufficient $CaCl_2$ to balance contained citrate) in buffer.

†Volume of urea solution equal to volume of system. Results duplicated with 60 per cent urea solution.

All other components as in previous protocol.

DISCUSSION

The observations made in the study of this case confirm previous studies in two similar cases.[6, 7] We agree with the general concept that the abnormal protein impairs the conversion of fibrinogen to fibrin. The data presented shed some additional light on the nature of this impairment.

The importance of the calcium ion in fibrinogen conversion has been re-emphasized by recent studies. The exaggerated circumstances of this case would support these data. The evidence that excess calcium improved the rate of fibrinogen conversion and changed the urea solubility of the fibrin clot indicates the impairment of calcium participation in the conversion of fibrinogen to fibrin in this instance.

The calcium-binding property of serum proteins is well known, although disagreement exists as to quantitation. Albumin exhibits the greatest binding capacity, although the globulins may bind equal amounts on a gram-for-gram basis. Rawson and Sunderman[23] reported that certain abnormal globulins in multiple myeloma exhibited normal or increased calcium-binding property, depending on the predominating electrophoretic globulin fraction present (the alpha globulins exhibiting increased binding property and the beta-gamma globulins normal or decreased). In addition, they found that the calcium-binding property of albumin in myeloma might increase, "in some cases to as much as 6 times the normal value." Although no data were obtained as to the calcium-binding capacity of the plasma proteins in this case, it is probable that interference with the participation of calcium in the clotting process was partly responsible for the impaired fibrin evolution. It is not known whether this interference was one of "calcium-binding" or some other phenomenon.

It is of interest that the calculated serum calcium of 10 mg. per cent and total protein of 10.0 Gm. per cent, according to the theoretical formulas of McLean and Hastings,[24] indicate an obligatory fraction of protein-bound calcium so relatively large as to leave a quite small amount of ionized calcium. Thus, assuming the calcium-binding capacity of the proteins to be normal, some 1.5 to 2.5 mg. per cent diffusible calcium would be present. The implications of this in regard to the role of ionized calcium in other body processes, such as neuromuscular irritability, cardiac function, and central nervous system function, are beyond the limits of this paper. However, no gross clinical changes were observed.

Whatever the mechanism of interference with calcium by the abnormal protein in the plasma and urine, it is believed that the abnormal gel formation in this case represented a form of fibrinogen conversion which was incomplete and possibly similar to the type of fibrin which forms in the absence of calcium and the serum factor. This concept may obviate the necessity of postulating a protective colloidal action of the abnormal protein blocking conversion of fibrinogen to fibrin at some intermediary point.[7]

The lack of gross interference with prothrombin conversion by the abnormal protein is not readily explained. Calcium is essential for prothrombin conversion by tissue thromboplastin, although the manner in which calcium participates in the reaction and whether ionized or protein-bound calcium is utilized in the physiologic state are undecided. In performing the one-stage prothrombin time it is our routine to add accelerator globulin in the form of bovine prothrombin-free plasma[25] to known dilutions of the plasma to be tested, and then add tissue thromboplastin and calcium. It is probable that the dilution, plus the addition of fibrinogen in the bovine plasma, in the presence of thromboplastin and additional calcium permitted a normal rate of prothrombin conversion. However, attempts to study prothrombin conversion in undiluted systems and by a modified two-stage procedure[26] failed to show definite impairment of prothrombin conversion. In the undiluted one-stage systems, slightly prolonged prothrombin times were obtained, but it was impossible to ascribe this prolongation to delayed prothrombin conversion in view of the known interference with fibrinogen conversion. The two-stage method in which another fibrinogen substrate was used yielded indefinite results because of the difficulty in defibrinating the patient's plasma with small amounts of thrombin. When the plasma was diluted, no impairment of prothrombin conversion was apparent, but, as previously noted, the effect of the abnormal protein on fibrin formation was also dissipated by dilution. Therefore, no conclusions can be drawn concerning the effect of the abnormal protein on thrombin production. The evidence available would not point to any impairment, an opinion which agrees with the findings of Uehlinger and Luescher and Labhart.

The finding that the urinary protein exhibited the same biologic properties as the plasma protein represents a unique relationship, to our knowledge, of the similarity of a plasma and urinary protein (''Bence-Jones'') affecting blood

clotting. Electrophoretically, the urinary protein was a single peak with a mobility similar to that in the plasma, although no data were obtained as to the molecular size and structure of the abnormal protein in blood and urine (studies are in progress). Uehlinger and Luescher and Labhart concluded that no single "macromolecule" was present in their cases. Rather, they considered the derangement represented a complex of gamma globulins. If this be true in our case, it is indeed interesting that such a complex could pass the renal filter and appear in the urine in an apparently unaltered state.

Emphasis should be placed on the absence of hemorrhagic or thrombotic phenomena in this patient. In previously described cases, hemorrhage first called attention to the disturbance of fibrinogen conversion. It may be that the disturbance was of greater degree in these cases than in ours. It is not difficult to visualize how the abnormal gel might represent an inadequate end stage of clotting by virtue of its slow formation, defective adhesiveness, and retraction. The abnormal gel or "profibrin" could alter platelet function, according to the concept that platelet adhesiveness is related to fibrin coating of the platelets.[10, 11]

It is doubtful that defective clot retraction is of major significance as a cause of impaired hemostasis. Budtz-Olsen, in his comprehensive review,[27] concludes that no data have been presented to show that defective hemostasis results from deficient clot retraction. It is clear that, in our case, gross impairment of clot retraction and delayed fibrinogen conversion did not give rise to hemorrhagic manifestations. Therefore, caution is imperative in ascribing impaired hemostasis to this or similar defects.

The authors wish to make clear their feeling that the mechanism of clot interference described in this case is an unusual variation in the disturbances of proteins in multiple myeloma. Whether such a disturbance occurs with any frequency in the disease depends upon the findings of future investigation.

SUMMARY AND CONCLUSIONS

A case of multiple myeloma is described in which an abnormality of plasma proteins was associated with impaired fibrinogen conversion. Visually, this was manifested by a total gelling of the blood or plasma without development of fibrin strands. This case is similar to two previously reported.[6, 7]

The abnormal protein appeared to exert its influence upon the conversion of fibrinogen by thrombin, preventing complete fibrin formation. The protein had similar effects on the fibrinogen of normal plasma and on isolated fibrinogen. It was found that the interference by the abnormal protein could be largely overcome by the addition of an excess of calcium ions to the clotting system. The resultant clot with calcium in the system formed more rapidly and had the physical characteristics of fibrin. The clot which formed in the presence of excess calcium was insoluble in 30 per cent urea solution, whereas, if calcium were omitted, the clot was soluble. The evidence supports previous reports that

calcium is essential for final fibrin formation. It is assumed that the abnormal gel in this patient's blood represented an intermediary form of the fibrinogen-fibrin transition similar to that described by Laki and Lorand and others.

The partial explanation is offered that the impaired fibrinogen conversion to thrombin lay in deficient availability of calcium for clotting due to interference by the abnormal protein. This may have been a reflection of the high calcium-binding property of the abnormal protein, although no data are presented to establish this.

The same interference was demonstrated by the protein found in the urine. This protein was electrophoretically homogeneous and of the same mobility as that of the plasma. Attenuation of the deleterious effect of the abnormal protein on blood clotting by excess calcium was noted for both the blood and urinary protein. It is believed they are the same protein or protein complex.

The literature indicating the relationship of the calcium ion to fibrin formation is briefly reviewed and the implications of our data in regard to this role of calcium are discussed. The possible importance of these findings to the occasional hemorrhagic diathesis in multiple myeloma is discussed.

Acknowledgments.—The authors gratefully acknowledge the assistance of Mrs. Hans Jordan in translations and the technical assistance of Miss Mary Baker, Senior Laboratory Technician, U. C. L. A. School of Medicine.

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INTRINSIC FACTOR STUDIES

II. The Effect of Gastric Juice on the Urinary Excretion of Radioactivity After the Oral Administration of Radioactive Vitamin B_{12}

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THE simultaneous oral administration of normal human gastric juice and small quantities of vitamin B_{12} results in hematopoiesis in patients with pernicious anemia in relapse, while oral vitamin B_{12} alone in similar amounts is usually ineffective.[1, 2, 3, 4, 8] The hematopoietic activity of intravenously administered vitamin B_{12} was not enhanced by the simultaneous intravenous administration of sterile Berkefeld filtered gastric juice in amounts known to be active as intrinsic factor when given orally.[5] Vitamin B_{12} by parenteral injection regularly corrects the hematologic abnormalities and arrests the neurologic disorder in pernicious anemia. Heinle and associates[6] have reported that the fecal excretion of radioactivity in four pernicious anemia patients receiving 0.5 μg of radioactive vitamin B_{12} ($B_{12}Co^{60}$) by mouth was definitely greater than in normal subjects. They reported that the simultaneous oral administration of an intrinsic factor preparation and $B_{12}Co^{60}$ in three pernicious anemia patients was followed by a definite reduction of fecal radioactivity. The simplest interpretation of the above information is that the function of intrinsic factor is to promote the absorption of vitamin B_{12} from the intestinal tract.

This paper reports observations on the effect of orally administered normal human gastric juice upon the quantity of radioactivity in the urine of pernicious anemia patients who were given a small oral dose of radioactive vitamin B_{12}.[9]

METHODS

A single lot of vitamin B_{12} containing radioactive cobalt (Co^{60}) and having a specific activity of 245 microcuries per milligram (μc/mg.)* was used as the tracer vitamin. In a windowless gas-flow Geiger counter this material contained approximately 280,000 counts per minute per microgram (c/m/μg). Urine samples were collected for twenty-four hours after the oral administration of radioactive vitamin according to the plan detailed below, and the radioactivity in the urine was determined by evaporating 2 ml. to dryness in a cup-type sample pan and counting in a windowless flow counter. All urine samples were counted in duplicate, and the data presented are an average of the two. When agreement between the duplicate counts was not close, two more samples were dried and counted.

After voiding the control specimen of urine, fasting subjects were given 2 μg of $B_{12}Co^{60}$ in fluid by mouth at 8:30 A.M., and one hour later they ate breakfast of coffee, toast, and fruit juice. Two hours after the *oral dose of radioactive* vitamin B_{12} each patient was given a *subcutaneous injection of 1000 μg of nonradioactive* vitamin B_{12}.† Total urine

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This investigation was supported by a research grant (A-69) from The National Institute of Arthritis and Metabolic Diseases of The National Institutes of Health, Public Health Service.

Presented before the Twenty-Sixth Annual Meeting of the Central Society for Clinical Research, Chicago, Ill., Oct. 30-31, 1953.

Received for publication July 20, 1953.

*Kindly furnished by Dr. Chas. Rosenblum, Merck and Company, Inc., Rahway, N. J.

†Kindly furnished by Dr. R. A. Peterson, Merck and Company, Inc., Rahway, N. J.

volumes were collected at 10:30 A.M., 12:30 P.M., 2:30 P.M., 4:30 P.M., 8:30 P.M., and 8:30 A.M., so that the radioactivity excreted in twenty-four hours could readily be calculated. Lunch and dinner were allowed as usual. The pernicious anemia patients had all been seen in relapse at one time or another, but when the present observations were made all were in satisfactory hematologic remission because of therapy.

Human gastric juice was collected, neutralized, and filtered as described previously.[10] Only juice secreted in response to histamine was utilized in this study.

When boiled gastric juice was used it was placed in a bath of boiling water for ten minutes and cooled before the $B_{12}Co^{60}$ was added. An exception was the observation of May 13, 1953, in patient A, when a mixture of $B_{12}Co^{60}$ and gastric juice was placed in boiling water.

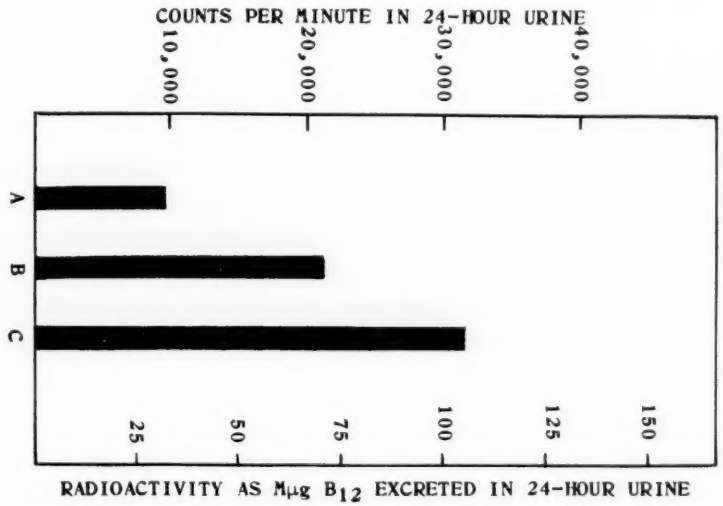

Fig. 1.—Urinary excretion of radioactivity in three normal persons given 2 μg $B_{12}Co^{60}$ orally, and two hours later, 1000 μg nonradioactive B_{12} subcutaneously.

RESULTS

Six normal people all excreted definite amounts of radioactivity in the urine when 2 μg of radioactive vitamin B_{12} were administered orally in 100 ml. of water and 1,000 μg of nonradioactive B_{12} were given subcutaneously under the conditions described above. Fig. 1 shows the urine radioactivity in 3 normal, young, control subjects. Data on 3 other normal persons are not pictured because urine collections were halted at the end of six to eighteen hours, at which time an easily detectable amount of radioactivity was being excreted in the urine. Six patients with pernicious anemia in remission excreted no detectable radioactivity in their urine when the $B_{12}Co^{60}$ was administered by mouth with water or boiled gastric juice. When in a different test period in each patient active gastric juice was administered with the oral $B_{12}Co^{60}$, each excreted easily detectable radioactivity in the urine beginning about four to six hours after the oral dose. The data are shown in Fig. 2 and Table I. Plasma samples showed no detectable radioactivity.

In the observation of April 17, 1953, on patient D, active gastric juice was given twelve hours after the oral $B_{12}Co^{60}$, and ten hours after the injection of nonradioactive vitamin B_{12}. No radioactivity appeared in the urine for thirty-six hours after the oral $B_{12}Co^{60}$.

The lack of effect of oxytetracycline (Terramycin) upon urine radioactivity is documented in Table I, patient C: observations dated May 1, 1953, and May 5, 1953, were conducted while the patient was receiving 250 mg. of Terramycin orally every six hours; the antibiotic administration was begun on April 27, 1953. It will be noted that no radioactivity appeared in the urine when $B_{12}Co^{60}$ was given in water (May 1, 1953) but when given in active gastric juice (May 5, 1953) definite radioactivity appeared in the urine.

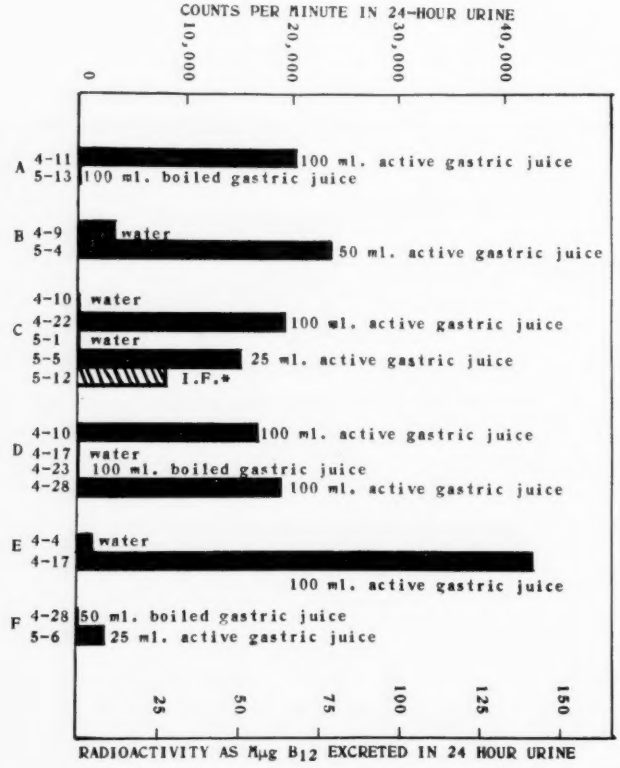

Fig. 2.—Urinary excretion of radioactivity in six pernicious anemia patients given 2 μg $B_{12}Co^{60}$ orally with gastric juice or water. 1000 μg nonradioactive B_{12} was given subcutaneously in each observation two hours after the oral $B_{12}Co^{60}$.

*I.F. = 100 mg. Intrinsic Factor Concentrate prepared from hog duodenum.

Observations on some of these and other subjects showed that when the 1,000 μg injection of nonradioactive vitamin B_{12} was omitted no radioactivity was detected in the urine of normal persons given oral $B_{12}Co^{60}$, or pernicious anemia subjects given normal human gastric juice and $B_{12}Co^{60}$ by mouth.

TABLE I. EFFECT OF GASTRIC JUICE UPON URINE RADIOACTIVITY IN PERNICIOUS ANEMIA PATIENTS GIVEN RADIOACTIVE VITAMIN B_{12} BY MOUTH AND NONRADIOACTIVE B_{12} BY PARENTERAL INJECTION

PATIENT	DATE	ORAL $B_{12}Co^{60}$ MICROGRAMS	NORMAL HUMAN GASTRIC JUICE† (ML.)	RADIOACTIVITY IN URINE COUNTS/MINUTE/ML. HOURS AFTER ORAL $B_{12}Co^{60}$						
				CONTROL	0-2	2-4	4-6	6-8	8-12	12-24
A	4/11/53	2	100	3	1	5	19	22	42	15
	5/13/53	2	100 (Boiled)§	2	4	3	1	2	2	0
B	4/ 9/53	2	0*	2	2	3	2	2	4	1
	5/ 4/53	2	50	1	1	2	26	30	24	30
C	4/10/53	2	0*	2	0	2	1	0	4	1
	4/22/53	2	100	5	4	6	18	46	31	26
	5/ 1/53	2	0*	2	1	1	2	2	2	3
	5/ 5/53	2	25	0	1	3	8	23	36	13
	5/12/53	2	I.F.‖	2	2	1	3	11	10	9
D	4/10/53	2	100	3	1	2	19	35	26	17
	4/17/53	2	100‡	3	1	1	1	2	2	0
	4/23/53	2	100 (Boiled)	3	2	3	2	2	1	1
	4/28/53	2	100	3	3	2	13	20	20	15
E	4/ 4/53	2	0*	1	0	0	2	3	3	1
	4/17/53	2	100	1	5	4	29	27	36	13
F	4/29/53	2	50 (Boiled)	1	2	0	2	5	2	2
	5/ 6/53	2	25	1	2	5	11	1	5	5

*Water used to dilute $B_{12}Co^{60}$.
†Given with oral $B_{12}Co^{60}$ except ‡ which was given twelve hours later.
§$B_{12}Co^{60}$ and gastric juice mixed and placed in boiling water for ten minutes.
‖100 mg. Intrinsic Factor concentrate from hog duodenum.

DISCUSSION

Progress toward the isolation of Castle's intrinsic factor has been hampered by the necessity of a tedious bioassay in patients with pernicious anemia in relapse. Heinle and co-workers[6] observed that pernicious anemia patients in remission who were given $B_{12}Co^{60}$ by mouth simultaneously with intrinsic factor excreted less radioactivity in the stool than when the radioactive vitamin was given without intrinsic factor. The observation was regarded as a new tool for the study of intrinsic factor activity. Meyer[7] has reported data somewhat at variance with those of Heinle and co-workers, but the oral dose of $B_{12}Co^{60}$ administered by Meyer was 1.0 μg, while Heinle and collaborators gave only 0.5 μg. The larger quantity given by Meyer may have obscured the effect of intrinsic factor. Preliminary data collected by Ley[11] show that the simultaneous oral administration of intrinsic factor does cause a decrease in fecal radioactivity in totally gastrectomized patients given radioactive B_{12} by mouth.

Using microbiological methods several observers[12, 13] have failed to demonstrate increased plasma or urine levels of vitamin B_{12} after oral administration of the vitamin to normal subjects or patients with pernicious anemia.

Conley and associates[12] and Mollin and Ross[14] have reported that as the parenteral dose of vitamin B_{12} is increased the percentage of that dose appearing in the urine in the following eight to twelve hours is increased. At the level of 1,000 μg of vitamin B_{12} subcutaneously approximately 80 to 100 per cent of the dose appeared in the urine in twenty-four hours. Because of these findings it occurred to me that a large parenteral injection of nonradioactive vitamin B_{12} might enhance the per cent excretion of radioactive B_{12}. This indeed

appears to be the case, for urine radioactivity was easily detected in normal subjects who received *oral* $B_{12}Co^{60}$ and a saturating parenteral injection of *nonradioactive* B_{12}. Normal subjects who were not given the parenteral vitamin excreted no detectable radioactivity in the urine after an oral dose of 2 μg of $B_{12}Co^{60}$.

The data offer no direct evidence as to the form in which the Co^{60} is excreted by the kidney. A sample of the radioactive urine from one of the control subjects was extracted with N-butanol and the radioactivity in the butanol extract was approximately 60 per cent of that present in the original sample. When $B_{12}Co^{60}$ was added to normal urine and the mixture extracted with N-butanol, a similar percentage of the radioactivity appeared in the extract. The fact that radioactivity is excreted in the urine only when the kidneys are excreting large quantities of nonradioactive vitamin B_{12} suggests that the radioactivity is in a molecule which is in competition with the nonradioactive B_{12} for the renal reabsorptve mechanisms. An attractive alternative explanation is that the nonradioactive B_{12} given by parenteral injection saturates the plasma binding capacity for B_{12} and, therefore, the $B_{12}Co^{60}$ absorbed from the gut is not bound to the plasma protein and hence appears in the glomerular filtrate. Mollin and Ross[14] have reported some observations on the ability of serum to combine with vitamin B_{12} to render the vitamin unavailable to certain microbes. In another study[10] paper electrophoresis of a mixture of serum and $B_{12}Co^{60}$ demonstrated that some of the vitamin moved with albumin fraction while the other part of the vitamin moved as it did when subjected to paper electrophoresis in saline solution.

The only known variable in the observations reported in this paper was the presence or absence of active gastric juice in the upper gastrointestinal tract with the $B_{12}Co^{60}$. Castle and Ham[15] have reported that when a twelve-hour lapse occurred between the administration of the extrinsic and the intrinsic factors no hemopoietic effect was observed in patients with pernicious anemia in relapse. In the present study, when active gastric juice was given 12 hours after the oral dose of $B_{12}Co^{60}$ and ten hours after the parenteral nonradioactive B_{12}, no radioactivity appeared in the urine.

In the observation dated May 12, 1953, in patient C, 100 mg. of intrinsic factor concentrate* prepared from hog duodenum was given with the $B_{12}Co^{60}$, and definite radioactivity appeared in the urine.

The observation of May 13, 1953, on patient A is of interest relative to the recent report of Spies and co-workers[16] that a boiled mixture of intrinsic factor and vitamin B_{12} was hemopoietic in two patients with "typical pernicious anemia." Unfortunately they did not publish data showing whether or not these patients had failed to respond to a similar oral dose of vitamin B_{12} without intrinsic factor. In a single observation in the present study a mixture of 2 μg of $B_{12}Co^{60}$ and 100 ml. of gastric juice was placed in boiling water for ten minutes. When the boiled mixture was administered to patient A on May 13, 1953, no

*Kindly supplied by Dr. K. C. Robbins, Armour Laboratories, Chicago, Ill.

detectable radioactivity appeared in the urine. However, when this patient ingested 2 μg $B_{12}Co^{60}$ with 100 ml. of unboiled gastric juice, the urine contained significant amounts of radioactivity.

Patients with pernicious anemia are reported to have a large number of bacteria in the gastro-duodenal contents,[17] and one theory of the mechanism of the vitamin B_{12} deficiency is that these organisms are effectively competing with the host for dietary vitamin B_{12}. Lichtman and associates[18] have reported data from four patients showing hemopoietic responses to oral Aureomycin. In one patient Ungley[3] found that a single 80 μg oral dose of vitamin B_{12}, after partial sterilization of the intestinal contents with antibiotics, was not followed by a hemopoietic response. However, in a subsequent observation in this patient, definite erythropoietic response ensued when 80 μg B_{12} and 500 ml. gastric juice were given by mouth. In the present study observations made on May 1, 1953, and May 5, 1953, on patient C (Table I and Fig. 1) demonstrate that radioactivity does not appear in the urine of a Terramycin treated pernicious anemia patient given oral $B_{12}Co^{60}$ unless normal human gastric juice is also administered. This observation, like that of Ungley, provides no support for the theory that intestinal microorganisms are depriving the host of dietary vitamin B_{12}.

SUMMARY AND CONCLUSIONS

Normal human gastric juice given simultaneously with an oral dose of radioactive vitamin B_{12} enhances the urinary excretion of radioactivity in pernicious anemia patients. This radioactivity did not appear in the urine unless the patient was given a subcutaneous "flushing" dose of 1,000 μg of nonradioactive vitamin B_{12}. Because the nonradioactive vitamin B_{12} made radioactivity appear in the urine, it is presumed that the radioactivity represents vitamin B_{12} or a closely related metabolite. The data support the theory that Castle's intrinsic factor enhances the intestinal absorption of vitamin B_{12} when given to patients with pernicious anemia.

This observation provides a new method for the study of intrinsic factor activity because observations can be made on pernicious anemia patients in hematologic remission.

It was the author's privilege to have the advice of Dr. Frank C. Larson on the technical aspects of quantitating the radioactivity in the specimens. Mrs. Ethel Slotnik and Mrs. Edith Eisenhauer provided valuable technical assistance.

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DISTRIBUTION OF COLLOIDAL RADIOACTIVE CHROMIC PHOSPHATE AFTER INTRACAVITARY ADMINISTRATION IN THE RAT

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THE distribution of intravenously injected radioactive colloids has been studied by many investigators.[1-4] The colloids were rapidly removed from the circulation and deposited in organs rich in reticulo-endothelial elements. Because of the current interest in the use of radioactive colloids in the irradiation of the peritoneal and pleural cavities, we have investigated the distribution of chromic phosphate colloids of varying size following intracavitary administration in the rat.

METHODS

Chromic Phosphate.—The radioactive chromic phosphate* was prepared by a modification of the method of Jones and co-workers.[1] Preparations with predominate particle sizes of 0.2 micron and 2 to 4 micra were prepared by variation of the grinding times. Studies were made on unstabilized colloid and on colloid stabilized with 2 per cent pectin or 5 per cent gelatin. Photographs taken with the electron microscope of three of the preparations are shown in Fig. 1. The unstablilized preparations show a striking tendency to form aggregates. Although the stabilizers used decreased, they did not completely prevent the tendency for aggregation to occur.

Animals.—White female rats of the Sprague Dawley strain were used. Their weights varied from 177 to 270 grams. The radioactive colloid was diluted to a concentration of approximately 6 microcuries per milliliter with 0.85 per cent saline, and 5 ml. were injected intraperitoneally. For the intrapleural experiments 1 ml. of the colloid containing 30 microcuries of P^{32} was injected in the left pleural space after preliminary injection of 1 ml. of air.

The animals were placed in metabolic cages for collection of urine. Two weeks later the animals were sacrificed with sodium pentobarbital, and samples of tissue were obtained for assay of the P^{32} content.

To avoid contamination by radioactive colloid deposited on the peritoneal surface, care was taken to exclude the surface of organs in obtaining samples for assay of radioactivity.

Assay Procedure.—Tissues were homogenized, using a glass homogenizer. Aliquots were plated in cupped planchets and slowly dried in an oven at 60° to 80° C. Recovery of radioactive chromic phosphate added to homogenates of tissues from normal animals was found to be from 97 to 101 per cent. Diluted urine samples were pipetted directly into cupped planchets and dried. The radioactivity was estimated by counting with an end window Geiger-Mueller counter for a period sufficiently long to reduce the statistical error of counting to 2 per cent or less. All results were corrected for decay.

RESULTS

The results obtained in the study of 21 animals are presented in Table I. In most cases four animals were used for each preparation. Two were injected

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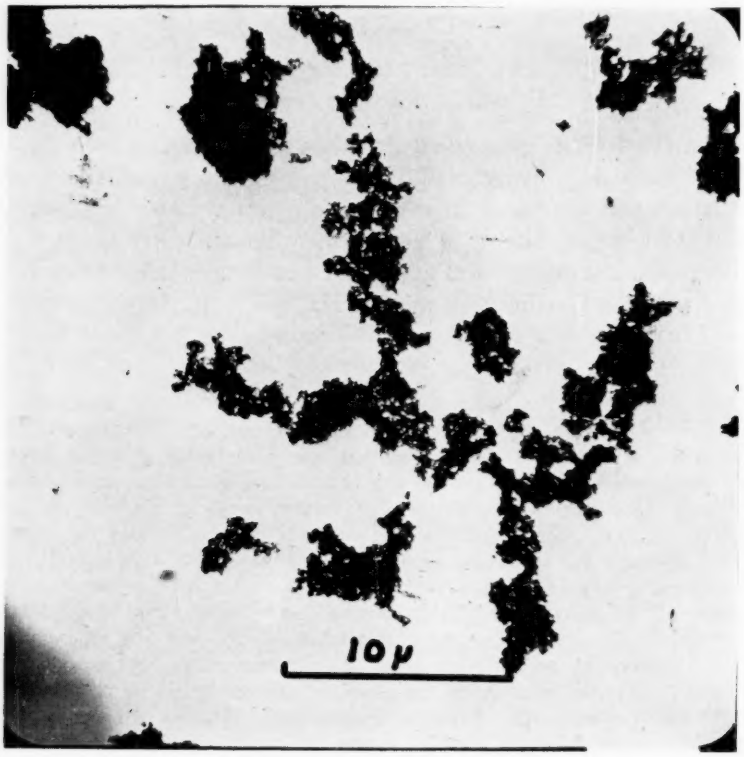
Received for publication, July 14, 1953.

*The preparations of chromic phosphate and electron micrographs were supplied by D. L. Tabern, Abbott Laboratories, North Chicago, Ill.

intraperitoneally and two intrapleurally. The averaged values are given as per cent of the dose per gram wet weight of tissue and per entire organ. The agreement between two animals injected with the same preparation was good. The total urinary excretion for the 14 day period is also given.



A.

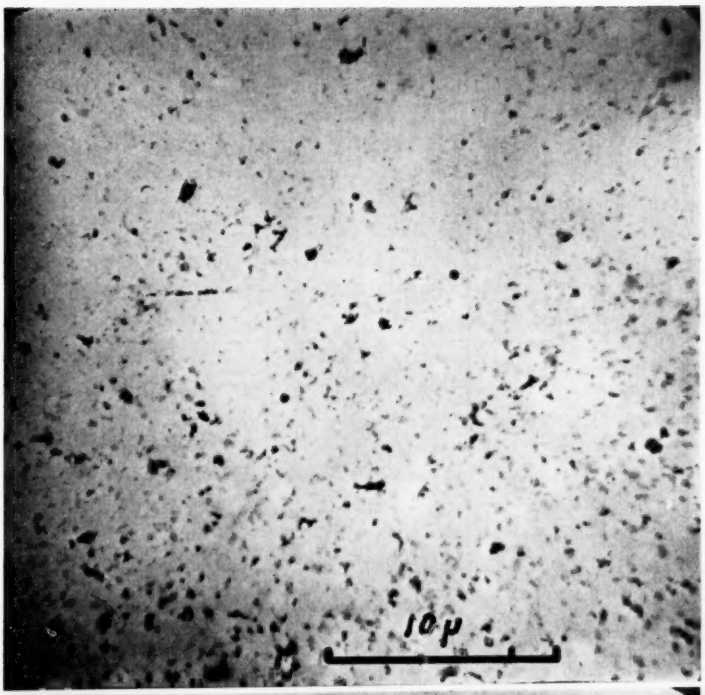
Fig. 1.—Electron micrographs of chromic phosphate preparations: *A*, 0.2 micron particles, unstabilized; *B*, 0.2 micron particles stabilized with pectin; *C*, 2 micra particles stabilized with pectin.

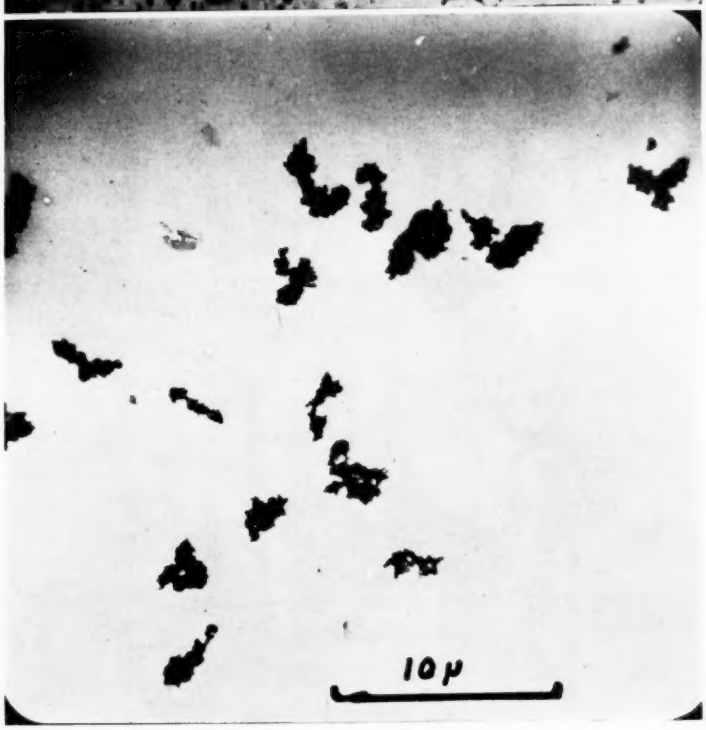
DISCUSSION

It is apparent that the 0.2 micron particles are much more readily removed from the pleural and peritoneal cavities than the larger particles. The addition of a stabilizing agent to the colloidal sol enhances its removal rate. This may be due to the greater tendency of the unstabilized colloids to aggregate and form large particles as demonstrated by the electron microscope studies. The larger aggregates would be expected to be removed more slowly. The type of surface coating may also affect the rate of removal. Zilversmit and his coworkers[2] found that the coating on colloidal gold particles influenced the rate of removal after intravenous administration.

In general, the various organs studied contained a higher percentage of the radioactive colloid after intraperitoneal than after intrapleural administra-

B.





C.

Fig. 1, *B* and *C*. (*For legend, see opposite page.*)

TABLE I. DISTRIBUTION OF RADIOACTIVE COLLOIDAL CHROMIC PHOSPHATE FOLLOWING INTRACAVITARY ADMINISTRATION

PREPARATION	NUMBER ANIMALS	% DOSE/GRAM						% DOSE/ORGAN				
		LIVER	KIDNEY	SPLEEN	LUNG	MUSCLE	BONE MARROW	LIVER	KIDNEY	SPLEEN	LUNG	URINE
Intraperitoneal												
0.2 micron	2	3.34	0.023	2.53	0.037	0.023	0.220	25.8	0.038	1.52	0.059	3.51
0.2 micron gelatin	2	4.90	0.033	2.86	0.140	0.026	0.389	38.70	0.060	2.90	0.170	3.14
0.2 micron pectin	4	4.87	0.053	2.83	0.077	0.017	0.237	45.06	0.082	3.34	0.112	2.70
2-4 micron	2	0.15	0.009	0.87	0.012	0.012	0.028	1.34	0.015	0.80	0.018	1.36
2-4 micron pectin	2	1.48	0.017	1.99	0.178	0.020	0.064	12.86	0.027	1.49	0.201	3.83
Intrapleural												
0.2 micron gelatin	2	2.660	0.030	1.35	R 0.923 L 1.060	0.015	0.275	18.5	0.050	1.40	R 1.60 L 2.25	3.53
0.2 micron pectin	3	2.047	0.039	0.710	R 0.170 L 0.861	0.018	0.160	21.72	0.070	1.156	R 0.376 L 0.393	2.31
2-4 micron	2	0.233	0.014	0.22	R 0.150 L 5.30	0.013	0.029	1.78	0.022	0.23	R 0.090 L 3.09	1.40
2-4 micron pectin	2	0.166	0.018	0.20	R 0.083 L 0.682	0.033	0.034	1.33	0.028	0.158	R 0.044 L 0.302	3.41

tion. This may be due to the greater surface area of the peritoneal cavity. The unit concentration of colloid in the liver and spleen was approximately the same. The bone marrow concentration was about one-tenth that of the spleen and the lung; kidney and muscle showed smaller amounts. If one makes the assumption that the bone marrow constitutes 8 per cent of the body weight, total bone marrow content did not exceed 6 per cent of the administered dose in any animal. When calculated on the basis of total organ, there was approximately 15 times as much colloid in the liver as the spleen for all preparations, whether they were administered intraperitoneally or intrapleurally. Under the conditions of this experiment, size had little effect on the distribution of the colloid after it entered the blood stream. The ratios are in agreement with those reported for intravenously administered colloid chromic phosphate by Jones and his co-workers,[1] and Gabrieli[3] and for intravenously and intraperitoneally administered radiogold by Berg.[4] The urinary excretion was low in all experiments and may be due to exchange of phosphate in chromic phosphate as reported by Neukomm and his co-workers.[5]

The large concentration of the 0.2 micron colloidal chromic phosphate in the liver after intracavitary administration indicates that caution should be exercised in its use in human beings, because of danger of irradiation to the liver and spleen. Although the danger is apparently less with larger particles, their size and tendency to form aggregates may result in uneven irradiation within the cavity.

SUMMARY

1. The distribution and excretion of various preparations of radioactive colloidal chromic phosphate after intracavitary administration in the rat have been studied.

2. Colloid with particle sizes of 0.2 micron was found in large amounts in the liver after intrapleural and intraperitoneal administration. With larger particle size a smaller fraction appeared in the liver.

3. Urinary excretion was negligible in all animals studied.

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STUDIES OF LIVER FUNCTION TESTS.

IV. EFFECT OF REPEATED INJECTIONS OF SODIUM BENZOATE ON THE FORMATION OF HIPPURIC ACID IN PATIENTS WITH LIVER DISEASE

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IT IS now well established that about 98 per cent of benzoic acid ingested is excreted in combined form, and that the two metabolites resulting from its detoxification are hippuric acid and benzoylglucuronic acid. Herbivorous animals ordinarily excrete benzoic acid nearly entirely as hippuric acid, whereas carnivorous animals excrete hippuric acid and benzoylglucuronic acid in approximately equal parts. In man, as in most animals, benzoylglucuronic acid appears in the urine only when large doses of benzoate are given or when liver disease is present. Small doses, such as 2 Gm. or less, are excreted entirely as hippuric acid under normal conditions. Even with moderate doses, for example, 5 to 10 Gm., hippuric acid is formed and excreted without altering the level of nitrogen excreted.[1]

Three factors affecting the formation of hippuric acid in man are the availability of glycine, the dose of benzoate given, and the rate of conjugation of glycine and benzoate. Quick[2] showed long ago that the rate of glycine synthesis is a major factor governing and limiting the formation of hippuric acid. He also observed that benzoic acid is a stimulus for the synthesis of glycine, and that the greater the amount given the greater the amount of glycine formed. It is now known that the glycine is derived from both exogenous and endogenous stores of protein.[3] Giving benzoate decreases the plasma glycine level normally, and giving glycine before or with benzoate increases the output of hippuric acid.[4]

The mechanism of hippurate formation from benzoate was studied in rat liver homogenates by Cohen and McGilvery,[5] who showed that the conjugation of para-aminobenzoic acid and glycine required adenosine triphosphate (ATP) and Mg^{++}. Borsook and Dubnoff[6] suggested that a similar system works for hippuric acid synthesis from benzoic acid, presenting evidence that ATP is necessary. More recently, Chantrenne[7] showed that hippuric acid is formed from glycine, benzoic acid, and ATP in extracts of rat liver acetone powder; and the enzyme system is reactivated by coenzyme A after deactivation with Dowex-1. It thus appears that the conjugation of glycine and benzoate requires the presence of ATP, Mg^{++}, and possibly coenzyme A.

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Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

Received for publication, Aug. 3, 1953.

In the presence of liver disease decreased hippuric acid is formed and increasing amounts of benzoylglucuronide are found in the urine, as shown by Snapper and co-workers.[8, 9] In a group of fifteen patients with liver disease they recovered two-thirds of the dose (1.77 Gm. sodium benzoate intravenously) as hippuric acid in two hours.[10] This was only 15 per cent less than the amount recovered over a corresponding period in normal adults. The injected benzoate is thus excreted in a matter of a few hours in both normal and abnormal subjects, though the fraction excreted in the first hour by the latter is smaller.

With the foregoing information in mind we present the observations of the present report which suggest that in the presence of liver disease repeated injections of a standard small amount of sodium benzoate stimulates the formation of increased amounts of hippuric acid.

METHODS AND MATERIALS

Case Material and Procedure.—The standard intravenous hippuric acid test was performed on each of three successive days on twenty-three normal subjects and twenty-nine patients with established liver disease. Seventeen (59 per cent) of the latter had portal cirrhosis, four (14 per cent) had hepatitis, and eight (27 per cent) had miscellaneous diseases including hemochromatosis, biliary cirrhosis, fatty liver, metastatic carcinoma, and caval thrombosis. The disease was moderate or severe in three-fourths of these abnormal subjects. The normal cases were hospital patients with no evidence of liver disease or any other serious systemic disease.

The patients were fasting at the time of testing each morning. Sodium benzoate (1.77 Gm.) was injected intravenously and the urine was collected at the end of one hour. To insure brisk urine flow during this interval two glasses of water were drunk by each subject shortly before the benzoate was injected. On the second and third mornings the first urine specimens voided on arising were saved initially. These represent urine formed approximately sixteen to twenty-four hours after the preceding injection of benzoate. Hippuric acid determinations were carried out on these night urines for the first twelve abnormal subjects studied, and none was recovered in each instance. The determinations were discontinued thereafter.

The procedure used for quantitating hippuric acid in urine utilizes ammonium sulfate to enhance precipitation and titration with NaOH for measurement of the amount of acid, as described previously.[11, 12] Values are expressed in grams of hippuric acid excreted in one hour.

Analysis.—Three comparisons were made for each group by taking the differences between the second and first days, third and first days, and third and second days. For any given comparison the mean of the differences was calculated and its significance tested by means of a "t" test. The measures of variation listed in the text are standard deviations (SD).

RESULTS AND INTERPRETATIONS

The amount of hippuric acid excreted in one hour on each of the three consecutive days is recorded for the normal subjects in Table I and for the patients with liver disease in Table II. The normal cases excreted an average of 1.07 ± 0.21 Gm. hippuric acid on the first day, 1.09 ± 0.18 Gm. on the second day, and 1.06 ± 0.23 Gm. on the third day. The patients with liver disease excreted an average of 0.54 ± 0.17 Gm. hippuric acid on the first day, 0.60 ± 0.21 Gm. on the second day, and 0.68 ± 0.24 Gm. on the third day.

For each individual the differences between the amounts of hippuric acid excreted on the first and second days, the first and third days, and the second

TABLE I. HIPPURIC ACID EXCRETED IN ONE HOUR BY NORMAL SUBJECTS

FIRST DAY (GM.)	SECOND DAY (GM.)	THIRD DAY (GM.)
1.00	1.21	1.07
0.91	1.07	1.05
1.01	0.94	1.13
1.11	1.34	—
0.75	1.07	0.72
0.62	—	0.79
1.09	1.13	—
1.25	1.23	1.18
1.02	1.27	1.20
1.07	0.77	—
1.05	0.88	0.58
1.49	1.36	1.33
0.92	0.94	0.79
1.31	0.81	1.20
0.78	0.84	0.69
0.86	0.91	1.02
1.20	1.24	1.19
1.23	1.36	1.24
1.38	1.13	1.24
1.00	1.19	1.19
1.27	1.17	1.46
1.15	1.01	0.99
1.04	1.04	1.09

TABLE II. HIPPURIC ACID EXCRETED IN ONE HOUR BY PATIENTS WITH LIVER DISEASE

FIRST DAY (GM.)	SECOND DAY (GM.)	THIRD DAY (GM.)
0.57	0.60	0.72
0.47	0.42	0.71
0.82	0.93	1.09
0.40	0.43	0.52
0.88	0.93	1.20
0.39	0.39	0.35
0.41	0.45	0.55
0.55	0.66	0.74
0.76	0.52	0.55
0.48	0.66	0.69
0.62	0.58	0.71
0.33	0.35	0.52
0.55	—	0.75
0.73	0.75	0.81
0.31	0.77	0.94
0.68	—	1.16
0.65	0.65	0.72
0.30	0.42	0.36
0.66	0.94	0.96
0.48	0.31	0.46
0.75	0.91	0.68
0.70	0.96	0.82
0.60	0.76	0.82
0.26	0.30	0.27
0.32	0.40	0.45
0.73	0.66	0.62
0.52	0.54	0.34
0.37	0.45	—
0.37	0.47	0.48

and third days were calculated. Four-fifths of the abnormal patients formed increased amounts of hippuric acid on the second and third days. Table III lists the mean differences derived and the results of testing the significance of these mean differences. All of the day-to-day comparisons among those without

liver disease are insignificantly different. The abnormal patients, however, excrete significantly greater amounts of hippuric acid on each succeeding day. The largest difference is naturally between the first and third days. The contrast between the "t" values and probabilities recorded for the normal and abnormal subjects is particularly striking.

TABLE III. SIGNIFICANCE OF MEAN DIFFERENCES AMONG VARIOUS DAY-TO-DAY COMPARISONS, BY GROUP

COMPARISON	NO. OF CASES	MEAN DIFFERENCE	SD OF DIFFERENCES	t	P
Normal Subjects					
Second-First	22	0.001	0.20	0.02	>0.90
Third-First	20	−0.005	0.17	0.12	>0.90
Third-Second	19	−0.016	0.19	0.39	>0.70
Abnormal Subjects					
Second-First	27	0.07	0.14	2.50	<0.02
Third-First	28	0.13	0.18	3.86	<0.001
Third-Second	26	0.05	0.12	2.07	<0.05

COMMENT

This observation that a constant small dose of sodium benzoate repeated on successive days results in the formation of increased amounts of hippuric acid in patients with liver disease has several interesting implications, though its exact significance remains to be worked out. The stimulus of an initial dose of benzoate may be on the mechanism of formation of glycine or on the enzymatic conjugation system. Defects in both glycine formation and conjugation probably exist in the presence of liver disease, though there is some evidence that the former is more important. Probstein and Londe[13] administered sodium benzoate to thirty patients with liver dysfunction. They noted increased excretion of hippuric acid in four-fifths upon simultaneous glycine administration, suggesting that the defect had been defective formation of glycine. In contrast one-fifth of the group showed no increase in hippuric acid excretion upon administration of glycine, suggesting a defect in conjugation. Perhaps the observation that four-fifths of our abnormal group showed increased formation of hippuric acid between the first and third days is more than coincidence. Barker, Capps, and Allen[14] obtained normal hippuric acid excretion in 98 per cent of 156 patients with hepatitis upon simultaneous oral administration of benzoate and glycine. They noted, however, that the oral hippuric acid test frequently gave relatively normal results during the acute stage of hepatitis. Saltzman and Snapper[15] likewise found that hippuric acid excretion in patients with liver disease approached the normal when glycine was given before benzoic acid. Glucuronide excretion decreased simultaneously.

Quick[2] has shown that benzoic acid stimulates the synthesis of glycine. The present results, in the light of the above studies of patients with liver disease, suggest that an initial dose of benzoate "primes the pump" so that a greater response is obtained on repetition of the same stimulus. Presumably the primary effect is on glycine formation, though one cannot ignore the possibility of an effect on the conjugation system.

The absence of this priming effect in the normal subjects is probably related to the adequacy of their glycine synthesis. With a sufficiently large dose of sodium benzoate, one might anticipate the same phenomenon.

SUMMARY

The standard intravenous dose of sodium benzoate (1.77 Gm.) was given on each of three consecutive mornings to a group of normal subjects and a group of patients with liver disease, and the amount of hippuric acid excreted in one hour was measured.

The normal individuals formed approximately the same amount of hippuric acid on each of the three days.

Four-fifths of the abnormal patients formed increased amounts of hippuric acid on the second and third days. For the entire group, the average hippuric acid excretion rose significantly from 0.54 to 0.68 Gm. This phenomenon is most probably due to a priming effect of the initial dose of benzoate on the mechanism of glycine synthesis.

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ANTIBIOTIC COMBINATIONS AND RESISTANCE TO ANTIBIOTICS

Development of Resistance During Repeated Subcultures of Staphylococci and Certain Streptococci on Media Containing Penicillin, Streptomycin, Erythromycin, Terramycin, and Chloramphenicol Used Singly and in Pairs

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MOST of the recent interest in the use of combinations of antimicrobial agents has been focused on their synergistic or antagonistic action.[1-7] There have also been some studies on the effect of combinations of antibiotics on the development or emergence of antibiotic-resistant strains during the course of exposures of originally sensitive bacteria in vitro, or during the course of treatment of infections due to such bacteria.[6, 8-14] Some of the latter studies have been focused particularly on the tubercle bacillus[10, 12-14] and the use of the combination of streptomycin and para-aminosalicylic acid has been of particular importance clinically in delaying the emergence of streptomycin-resistant tubercle bacilli, thus permitting more prolonged and effective chemotherapy with these two agents.

In many large hospitals there is now also much concern over the fact that increasing proportions of staphylococci that are being isolated from infectious materials are proving to be resistant to penicillin and to some other antibiotics which are in common use.[15, 16] The recent demonstration that the new antibiotic, erythromycin, is highly active against staphylococci from various sources, irrespective of their sensitivity or resistance to other antibiotics,[17-20] has been tempered by the observation that staphylococci and some other organisms readily become resistant to erythromycin upon exposure to that agent either in vitro[21, 22] or during the course of treatment.[18, 23, 24] Studies in this laboratory have shown that in vitro the development of resistance by staphylococci to erythromycin and also to penicillin or streptomycin could be delayed and depressed by the use of a combination of erythromycin with either or the other 2 antibiotics.[25]

The present paper deals with an extension of these studies and includes observations on the development of resistance by strains of Staphylococcus, Enterococcus, and Streptococcus viridans to penicillin, streptomycin, chloramphenicol, Terramycin, and erythromycin during the course of repeated subcultures in these agents, used individually or in pairs. Some observations on the development of cross-resistance to other antibiotics, namely, Aureomycin, neomycin, and carbomycin, are also included.

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Aided by a grant from the United States Public Health Service.
Received for publication, June 30, 1953.

MATERIALS AND METHODS

The strains of bacteria used in this study were all originally isolated from infected material of patients in this hospital, either in this laboratory or by Miss Marion E. Lamb and her associates in the Bacteriology Laboratory of the Mallory Institute of Pathology. They were all derived from single colonies and were used either directly, after 2 or 3 subcultures, or after storage for varying periods at 5° C. or −25° C. and further subcultures. The staphylococcal strains were obtained either from blood cultures or from purulent materials; they were all hemolytic, coagulase positive, and produced the characteristic yellow pigment of *Staph. aureus*. The enterococci were obtained from urines of patients with pyelonephritis and were also hemolytic and resisted heating for 30 minutes at 56° or 60° C. The strains of *Strep. viridans* were isolated from the blood of patients with subacute bacterial endocarditis.

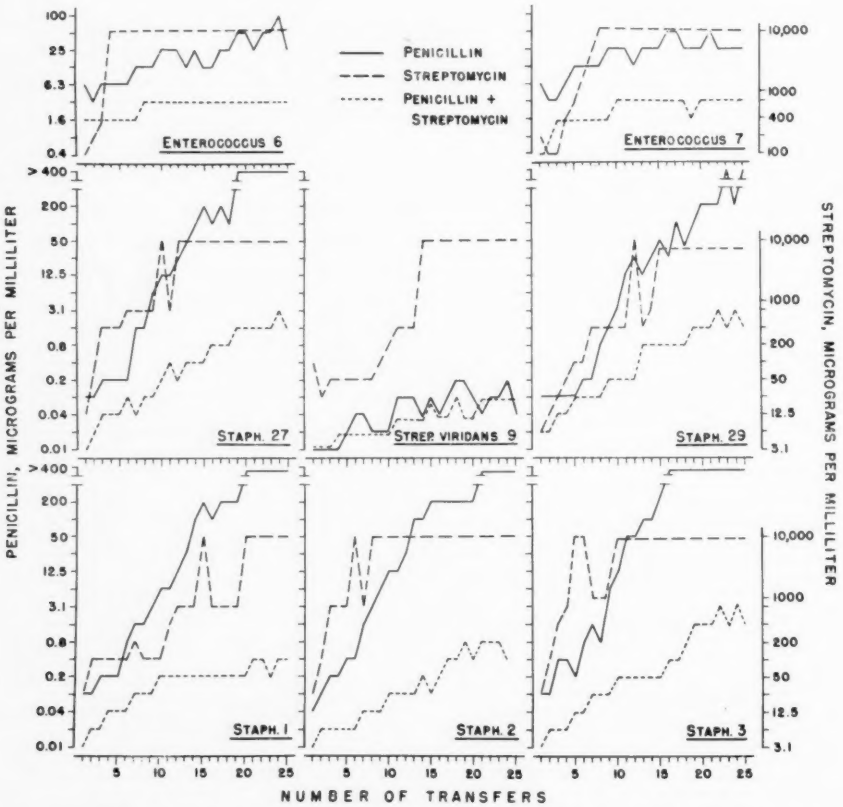

Fig. 1.—Changes in resistance to penicillin, streptomycin, and penicillin plus streptomycin during successive subcultures on blood agar containing increasing concentrations of these antibiotics, respectively.

Heart infusion agar (Difco) to which 10 per cent defibrinated horse blood was added was the basal medium used throughout, otherwise the materials and methods were similar to those employed in previous studies in this laboratory on the development of resistance and cross-resistance to antibiotics in vitro.[21, 22, 25-28] The choice of the strains used with each pair of antibiotics was determined largely by their initial sensitivity to these agents; for the most part the strains were chosen for each experiment because they were similar in their sensitivity to each of the antibiotics to be used. Each antibiotic mixture was used in a

constant ratio and this was the same for all of the strains studied with that mixture, except with the combination of penicillin and chloramphenicol; with the latter it was necessary to use the antibiotics in a different ratio for the strains of Enterococcus.

RESULTS

Observations were made with 6 pairs of antibiotics other than the combinations of erythromycin with penicillin and streptomycin which were previously reported.[25] With each pair of antibiotics, 8 or 9 strains were exposed to 4 parallel series of subcultures, one on antibiotic-free blood agar, the second and third on similar media containing graded concentrations of each of the individual antibiotics, and the fourth on graded concentrations of the combination—always present in a constant ratio. For each combination of drugs the successive changes in resistance of each of the strains to the individual antibiotics and to the pair on which they were subcultured will be depicted graphically.

TABLE I. EFFECT OF 25 SUBCULTURES ON AGAR CONTAINING PENICILLIN, STREPTOMYCIN, OR BOTH ON THE SENSITIVITY OF 8 BACTERIAL STRAINS TO THESE ANTIBIOTICS AND TO NEOMYCIN

STRAIN	NUMBER OF TRANSFERS*	MINIMUM INHIBITING CONCENTRATION, μG/ML.			
		PENICILLIN (P)	STREPTOMYCIN (S)	P + S†	NEOMYCIN
Staphylococcus 1	0 × 25	0.1	6.3	0.05 + 12.5	12.5
	P × 25	>400	3.1	0.05 + 12.5	0.4
	S × 25	0.05	>10,000	0.05 + 12.5	50.
	P + S × 25	0.4	200	0.4 + 100	6.3
Staphylococcus 2	0 × 25	0.05	25	0.05 + 12.5	1.6
	P × 25	>400	3.1	0.05 + 12.5	0.4
	S × 25	0.05	>10,000	0.05 + 12.5	12.5
	P + S × 25	12.5	1000	0.8 + 200	6.3
Staphylococcus 3	0 × 25	0.1	6.3	0.05 + 12.5	1.6
	P × 25	>400	12.5	0.1 + 25	0.8
	S × 25	0.05	>10,000	0.05 + 12.5	50.
	P + S × 25	12.5	10,000	1.6 + 400	6.3
Staphylococcus 27	0 × 25	0.05	25	0.05 + 12.5	1.6
	P × 25	>400	3.1	0.05 + 12.5	0.4
	S × 25	0.05	>10,000	0.05 + 12.5	50.
	P + S × 25	1.6	1000	1.6 + 400	50.
Staphylococcus 29	0 × 25	0.1	6.3	0.05 + 12.5	12.5
	P × 25	>400	3.1	0.05 + 12.5	0.4
	S × 25	0.05	>10,000	0.05 + 12.5	50.
	P + S × 25	3.1	1000	1.6 + 400	50.
Enterococcus 6	0 × 25	6.3	200	0.4 + 100	100
	P × 25	25	400	0.2 + 50	100
	S × 25	3.1	>10,000	1.6 + 400	800
	P + S × 25	12.5	10,000	3.1 + 800	800
Enterococcus 7	0 × 25	6.3	100	0.4 + 100	200
	P × 25	25	400	0.8 + 200	400
	S × 25	3.1	>10,000	1.6 + 400	800
	P + S × 25	3.1	10,000	3.1 + 800	800
Strep. viridans 9	0 × 25	0.01	25	0.01 + 3.1	
	P × 25	0.05	12.5		
	S × 25	0.05	>10,000		
	P + S × 25	0.2	50.	0.1 + 25	

*0 = no antibiotic.
†Constant ratio of concentrations in P + S (P:S = 1:256).

Although tests for cross-resistance were also carried out at intervals in the course of each experiment and after the twenty-fifth subcultures when the experiments were all arbitrarily terminated, only the results obtained at the end of the experiment with the strains of each of the 4 series of subcultures will be given and these will be presented in tabular form for each of the combinations studied.

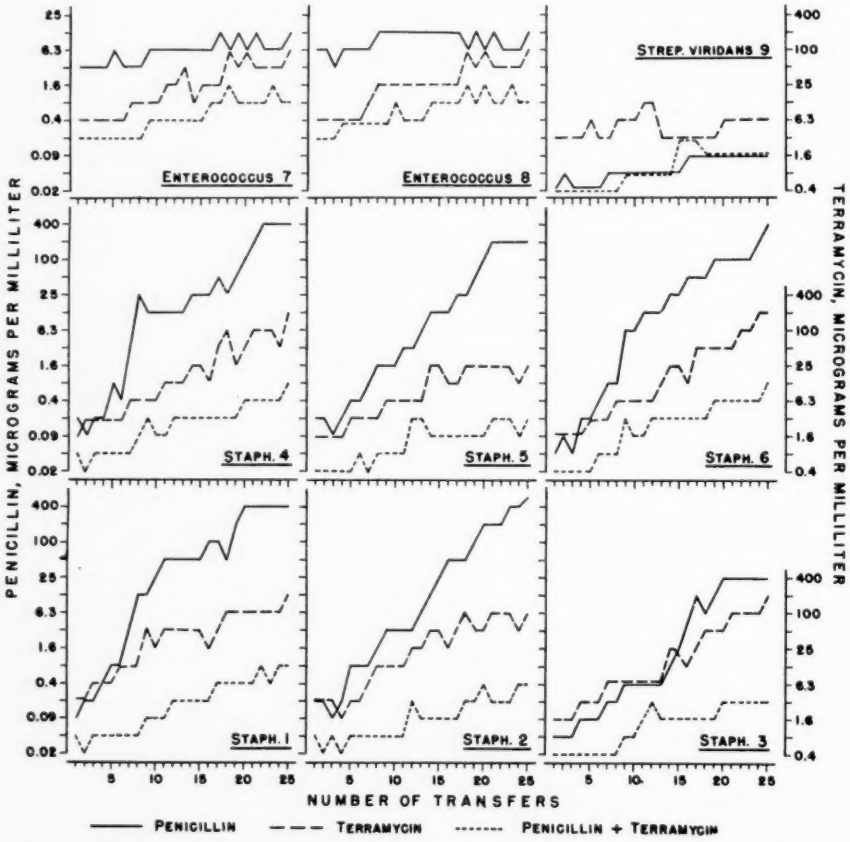

Fig. 2.—Changes in resistance to penicillin, Terramycin, and penicillin plus Terramycin during successive subcultures on blood agar containing increasing concentrations of these antibiotics, respectively.

Penicillin and Streptomycin.—When exposed to either penicillin or streptomycin the staphylococcal strains all increased in resistance to the homologous agent steadily and rapidly while the streptococci became rapidly resistant to streptomycin and increased slowly and only slightly in their resistance to penicillin (Fig. 1). Cross-resistance between penicillin and streptomycin did not develop, but 2 of the staphylococcal strains (Nos. 2 and 27) apparently increased in sensitivity to streptomycin following their exposures to penicillin. The sensitivity of the strains to the mixture of the 2 antibiotics was unaffected by the subcultures in the individual agents except that the enterococci showed

a 4-fold increase in resistance to the mixture following their exposures to streptomycin (Table I).*

The subcultures of the staphylococcal strains on the 1:256 mixture of penicillin and streptomycin resulted in a steady increase in resistance to that mixture, but at a slower rate as compared with the increases in resistance to the homologous agents when used individually. The streptococcal strains responded to the subcultures on the mixture in a manner similar to their response to penicillin (Fig. 1). Resistance to the individual components of the mixture in-

TABLE II. EFFECT OF 25 SUBCULTURES ON AGAR CONTAINING PENICILLIN, TERRAMYCIN, OR BOTH ON THE SENSITIVITY OF 9 BACTERIAL STRAINS TO THESE ANTIBIOTICS AND TO AUREOMYCIN

STRAIN	NUMBER OF SUBCULTURES*	MINIMUM INHIBITING CONCENTRATION, μG/ML.			
		PENICILLIN (P)	TERRAMYCIN (T)	P + T†	AUREOMYCIN
Staphylococcus 1	0 × 25	0.2	1.6	0.02 + 0.4	0.4
	P × 25	400.	0.8	0.02 + 0.4	0.2
	T × 25	0.2	200	0.04 + 0.8	12.5
	P + T × 25	3.1	25	0.8 + 12.5	3.1
Staphylococcus 2	0 × 25	0.2	1.6	0.02 + 0.4	0.4
	P × 25	400.	0.8	0.02 + 0.4	0.2
	T × 25	0.2	100	0.04 + 0.8	6.3
	P + T × 25	0.4	6.3	0.4 + 6.3	1.6
Staphylococcus 3	0 × 25	0.1	0.8	0.02 + 0.4	0.4
	P × 25	25.	0.8	0.02 + 0.4	0.2
	T × 25	0.2	200	0.04 + 0.8	12.5
	P + T × 25	0.4	6.3	0.2 + 3.1	1.6
Staphylococcus 4	0 × 25	0.2	1.6	0.02 + 0.4	0.4
	P × 25	400.	0.8	0.02 + 0.4	0.2
	T × 25	0.2	200	0.04 + 0.8	6.3
	P + T × 25	0.4	12.5	0.8 + 12.5	3.1
Staphylococcus 5	0 × 25	0.1	1.6	0.02 + 0.4	0.4
	P × 25	200.	0.8	0.02 + 0.4	0.2
	T × 25	0.2	25	0.02 + 0.4	6.3
	P + T × 25	0.8	6.3	0.2 + 3.1	0.8
Staphylococcus 6	0 × 25	0.1	1.6	0.02 + 0.4	0.4
	P × 25	400.	0.8	0.02 + 0.4	0.2
	T × 25	0.1	200	0.02 + 0.4	12.5
	P + T × 25	0.8	6.3	0.8 + 12.5	3.1
Enterococcus 7	0 × 25	3.1	12.5	0.1 + 1.6	3.1
	P × 25	12.5	6.3	0.1 + 1.6	0.8
	T × 25	1.6	100	0.2 + 3.1	12.5
	P + T × 25	3.1	100	0.8 + 12.5	6.3
Enterococcus 8	0 × 25	3.1	12.5	0.2 + 3.1	3.1
	P × 25	12.5	6.3	0.1 + 1.6	0.8
	T × 25	1.6	100	0.1 + 1.6	6.3
	P + T × 25	3.1	100	0.8 + 12.5	6.3
Strep. viridans 9	0 × 25	0.02	3.1	0.01 + 0.2	3.1
	P × 25	0.1	3.1	0.01 + 0.2	0.8
	T × 25	0.02	12.5	0.01 + 0.2	3.1
	P + T × 25	0.2	6.3	0.1 + 1.6	0.8

*0 = no antibiotic.
†Constant ratio P:T = 1:16.

*The sensitivities of the parent strains when tested at the end of each experiment often varied from two- to fourfold from those obtained at the start of the study; for brevity and to avoid confusion, the initial sensitivities, which may be seen in the figures, are not listed in the tables.

creased markedly in the staphylococcal strains; in the enterococci the resistance to streptomycin increased while the sensitivity to penicillin was unaffected following the subcultures on the mixture of these agents. The reverse was true in the case of the strain of *Strep. viridans.*

Resistance to neomycin increased slightly in the strains which had become resistant to streptomycin. The staphylococcal strains, however, appeared to increase in sensitivity to neomycin following their exposures to penicillin alone.*

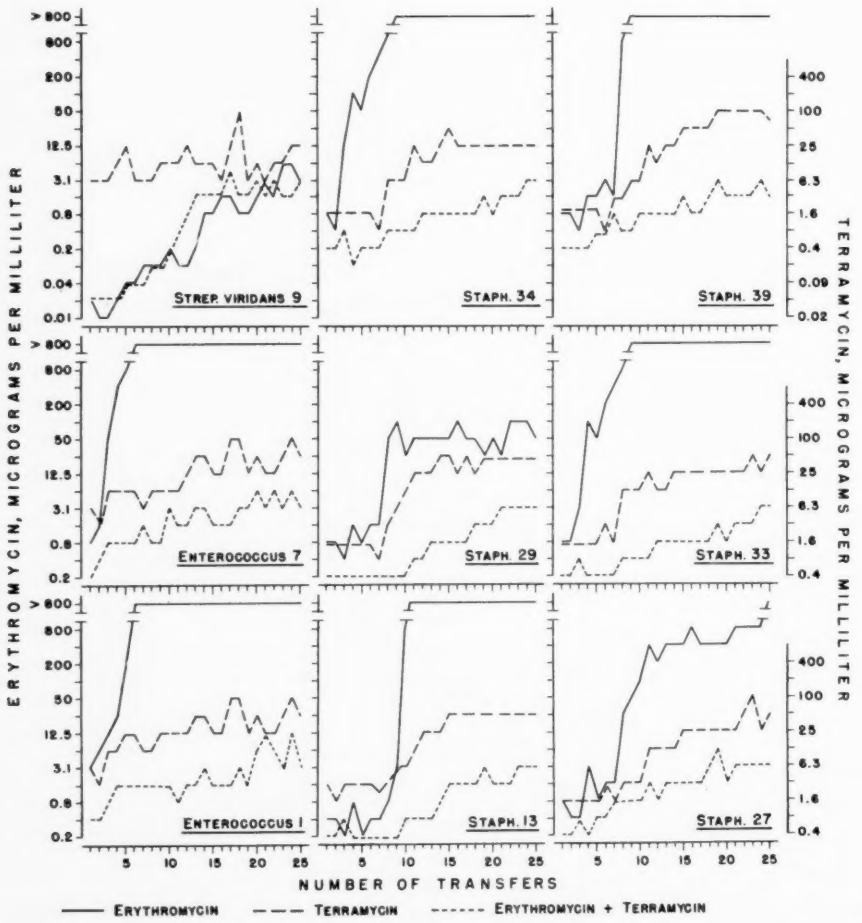

Fig. 3.—Changes in resistance to erythromycin, Terramycin, and erythromycin plus Terramycin during successive subcultures on blood agar containing increasing concentrations of these antibiotics, respectively.

Penicillin and Terramycin.—The effects of the subcultures on these 2 antibiotics, separately and together, on the resistance to the homologous antibiotics are shown in Fig. 2. The effects of penicillin alone were similar to those previously shown in Fig. 1, the differences observed with the same strains being well

*"Increase in sensitivity" means that less antibiotic is required to inhibit.

within the variations expected from the crude methods employed. The repeated subcultures in Terramycin alone resulted in increases in resistance to that antibiotic in all of the strains except *Strep. viridans* 9; for the staphylococci (except No. 3) these increases were at a distinctly slower rate and for the enterococci they were greater than the corresponding changes that occurred with penicillin alone. The repeated exposures to the mixture of these 2 antibiotics in a ratio of 1 part penicillin to 16 of Terramycin resulted in increases in resistance to this combination which were usually only slightly less than those observed with Terramycin alone.

TABLE III. EFFECT OF 25 SUBCULTURES ON AGAR CONTAINING ERYTHROMYCIN, TERRAMYCIN, OR BOTH ON THE SENSITIVITY OF 9 BACTERIAL STRAINS TO THESE ANTIBIOTICS AND ALSO TO CARBOMYCIN AND AUREOMYCIN

STRAIN	SUBCULTURES*	MINIMUM INHIBITING CONCENTRATION, μG/ML. ERYTHROMYCIN	TERRAMYCIN	ERYTHROMYCIN + TERRAMYCIN	CARBOMYCIN	AUREOMYCIN
Staphylococcus 13	0 × 25	0.4	0.8	0.2 + 0.4	6.3	0.4
	E × 25	>800.	3.1	0.2 + 0.4	>800.	0.4
	T × 25	0.4	25.	0.2 + 0.4	6.3	1.6
	E + T × 25	12.5	6.3	3.1 + 6.3	12.5	1.6
Staphylococcus 27	0 × 25	0.2	1.6	0.2 + 0.4	6.3	0.4
	E × 25	>800.	0.8	0.2 + 0.4	>800.	0.4
	T × 25	0.2	50.	0.2 + 0.4	1.6	3.1
	E + T × 25	12.5	6.3	3.1 + 6.3	12.5	1.6
Staphylococcus 29	0 × 25	0.4	1.6	0.2 + 0.4	6.3	0.4
	E × 25	50.	0.2	0.2 + 0.4	100.	<0.1
	T × 25	0.2	50.	0.4 + 0.8	6.3	3.1
	E + T × 25	50.	6.3	3.1 + 6.3	200.	1.6
Staphylococcus 33	0 × 25	0.4	1.6	0.2 + 0.4	6.3	0.4
	E × 25	>800.	3.1	0.4 + 0.8	>800.	0.4
	T × 25	0.4	50.	0.2 + 0.4	6.3	3.1
	E + T × 25	>800.	3.1	3.1 + 6.3	>800.	1.6
Staphylococcus 34	0 × 25	0.2	1.6	0.2 + 0.4	6.3	0.4
	E × 25	>800.	1.6	0.2 + 0.4	>800.	0.1
	T × 25	0.2	50.	0.4 + 0.8	3.1	3.1
	E + T × 25	12.5	6.3	1.6 + 3.1	50.	1.6
Staphylococcus 39	0 × 25	0.2	0.8	0.2 + 0.4	6.3	0.2
	E × 25	>800.	1.6	0.2 + 0.4	>800.	0.2
	T × 25	0.2	100.	0.4 + 0.8	6.3	6.3
	E + T × 25	400.	6.3	3.1 + 6.3	200.	1.6
Enterococcus 1	0 × 25	3.1	6.3	0.4 + 0.8	25.	6.3
	E × 25	>800.	6.3	0.2 + 0.4	>800.	12.5
	T × 25	3.1	50.	0.4 + 0.8	25.	50.
	E + T × 25	>800.	25.	3.1 + 6.3	200.	12.5
Enterococcus 7	0 × 25	0.8	6.3	0.2 + 0.4	12.5	12.5
	E × 25	>800.	6.3	0.4 + 0.8	>800.	12.5
	T × 25	1.6	50.	0.4 + 0.8	12.5	50.
	E + T × 25	400.	25.	3.1 + 6.3	12.5	50.
Strep. viridans 9	0 × 25	0.02	3.1	0.02 + 0.04	6.3	3.1
	E × 25	6.3	0.8	0.4 + 0.8	200.	3.1
	T × 25	0.01	25.	0.02 + 0.04	3.1	50.
	E + T × 25	400.	1.6	1.6 + 3.1	>800.	12.5

*0 = no antibiotic; E = erythromycin; T = Terramycin (ratio for combination was constant, E:T = 1:2).

From Table II it is seen again that the 25 exposures to penicillin and Terramycin, alone or in the 1:16 combination, resulted in increases in resistance to the homologous antibiotics, but these increases were small in the streptococci.

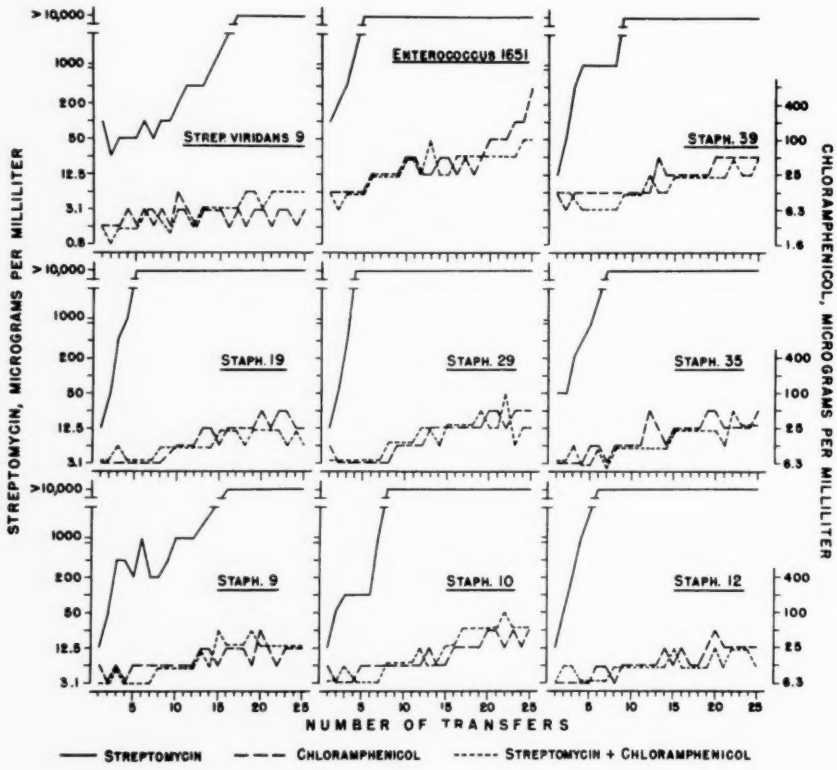

Fig. 4.—Changes in resistance to streptomycin, chloramphenicol, and streptomycin plus chloramphenicol during successive subcultures on blood agar containing increasing concentrations of these antibiotics, respectively.

Cross-resistance between penicillin and Terramycin was not observed, nor did any significant increase in sensitivity to either of these antibiotics occur as a result of the 25 subcultures on the other. Likewise, the sensitivity of each strain to the mixture was the same, within the error of the method, after the subcultures in either penicillin or Terramycin as they were after the parallel transfers on antibiotic-free media. After the 25 subcultures in the combination of these antibiotics, however, all of the strains increased in resistance to the individual antibiotics, and to about the same extent (usually 4- to 8-fold), except for the enterococci which did not increase in resistance to penicillin.

Erythromycin and Terramycin.—As shown in Fig. 3, each of the strains except Staphylococcus 29 and *Strep. viridans* 9 increased rapidly and markedly in resistance to erythromycin. The increases in resistance to Terramycin closely resembled those noted in the preceding experiment. Also, as in that experiment, the increase in resistance to the mixture resulting from subcultures in

the combination of erythromycin and Terramycin almost paralleled the increases noted with Terramycin alone, except in the case of *Strep. viridans* 9 in which the increases in resistance to the mixture paralleled that resulting from the exposures to erythromycin alone.

The strains of the twenty-fifth transfers of all 4 series (Table III) showed increases of varying degrees in resistance to the antibiotics on which they were grown; to erythromycin they increased more than 2000-fold, except for Staphylococcus 29 and *Strep. viridans*, which increased 128- and 256-fold, respectively; to Terramycin the staphylococci increased in resistance 32-fold or more and the streptococci 8-fold; and in the 1:2 mixture the increases were 16-fold for staphylococci, 8-fold for the enterococci, and 64-fold for *Strep. viridans* 9.

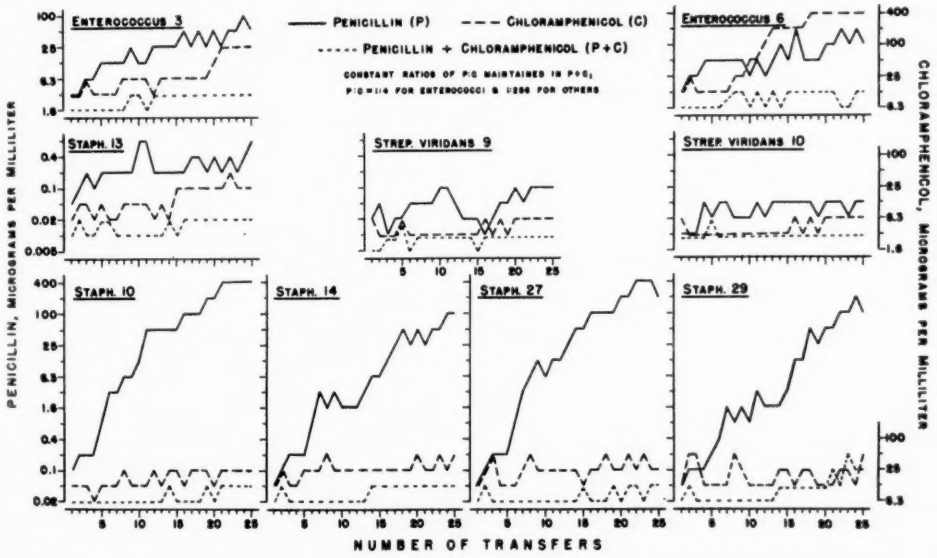

Fig. 5.—Changes in resistance to penicillin, chloramphenicol, and penicillin plus chloramphenicol during successive subcultures on blood agar containing increasing concentrations of these antibiotics, respectively.

Cross-resistance between erythromycin and Terramycin did not develop. Likewise, increases in sensitivity to one of these agents resulting from the subcultures in the other were not observed except in Staphylococcus 29 and *Strep. viridans* 9 which, after the 25 exposures to erythromycin, were inhibited by one-eighth and one-fourth, respectively, of the concentrations of Terramycin required to inhibit the corresponding control strains which had been subcultured on antibiotic-free media. Also, increases in resistance to the combination did not result from the subcultures on the individual antibiotics except for *Strep. viridans* 9 which increased 16-fold in resistance to the 1:2 mixture after the 25 subcultures on erythromycin. On the other hand, the strains which had been transferred 25 times on the combination of antibiotics all increased markedly (most of them 128-fold or more) in resistance to erythromycin and, except for the strain of *Strep. viridans*, they also increased slightly in resistance to Terramycin.

As in a previous study,[22] cross-resistance to carbomycin developed regularly and usually to a marked degree in all of the strains which had increased in resistance to erythromycin. Likewise, all of the strains which had been exposed to Terramycin, alone or in combination, exhibited some increase in resistance to aureomycin.

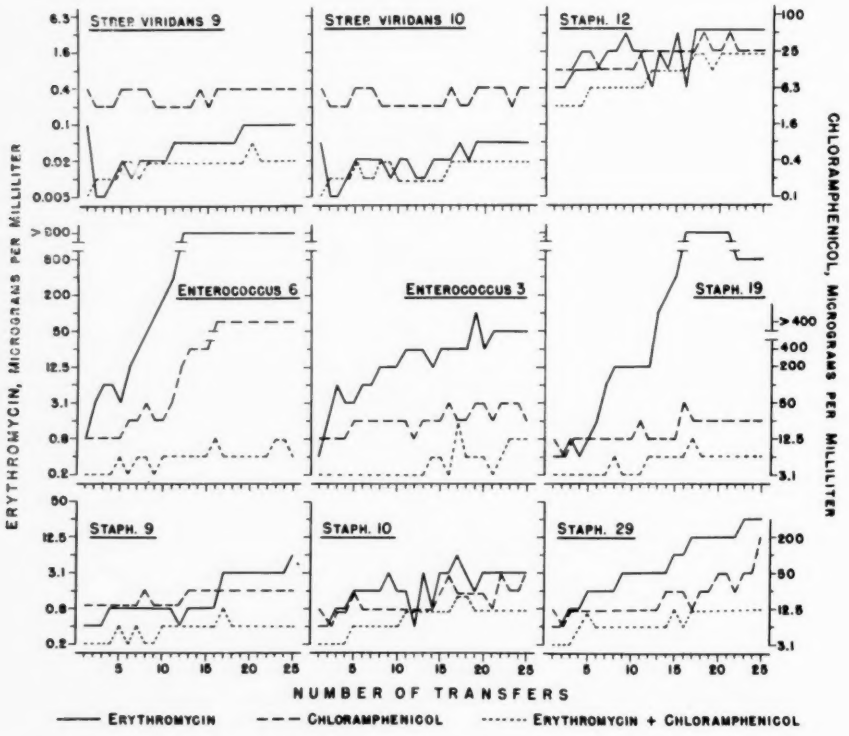

Fig. 6.—Changes in resistance to erythromycin, chloramphenicol, and erythromycin plus chloramphenicol during successive subcultures on blood agar containing increasing concentrations of these antibiotics, respectively.

Combinations With Chloramphenicol.—The results of 3 experiments in which chloramphenicol was paired with either streptomycin, penicillin, or erythromycin showed many similarities and will, therefore, be summarized together. The effects of the successive exposures to the antibiotics on the sensitivity to the homologous agents are depicted in Figs. 4, 5, and 6. (1) Subcultures on chloramphenicol alone, in each of the 3 experiments, resulted in moderate increases in resistance in that antibiotic in the strains of Enterococcus, but in only little or no increase in resistance in the strains of Staphylococcus or *Strep. viridans.* (2) Resistance to streptomycin increased rapidly in all strains (Fig. 4); resistance to penicillin (Fig. 5) and to erythromycin (Fig. 6) increased, though to varying degrees, in all strains except those of *Strep. viridans.* (3) During the subcultures on the mixtures, the increases in resistance to chloramphenicol plus streptomycin closely paralleled the increases in resistance to chloramphenicol when used alone (Fig. 4), but there were no significant increases in re-

sistance to the other 2 mixtures except in some of the staphylococci, which showed slight increases in resistance to the combination of chloramphenicol and erythromycin.

TABLE IV. EFFECT OF 25 SUBCULTURES ON AGAR CONTAINING CHLORAMPHENICOL, STREPTOMYCIN, OR BOTH ON THE SENSITIVITY OF 9 BACTERIAL STRAINS TO THESE ANTIBIOTICS AND TO NEOMYCIN AND PENICILLIN

STRAIN	NUMBER OF SUBCULTURES*	MINIMUM INHIBITING CONCENTRATION, μG/ML.				
		CHLORAMPHENICOL	STREPTOMYCIN	CHLORAMPHENICOL + STREPTOMYCIN	NEOMYCIN	PENICILLIN
Staphylococcus 9	0 × 25	12.5	12.5	6.3 + 3.1	12.5	0.8
	C × 25	25.	3.1	25 + 12.5	12.5	0.2
	S × 25	6.3	>10,000	12.5 + 6.3	50.	0.1
	C + S × 25	25.	25.	25 + 12.5	25.	0.1
Staphylococcus 10	0 × 25	12.5	25.	6.3 + 3.1	12.5	0.1
	C × 25	50.	12.5	12.5 + 6.3	6.3	0.2
	S × 25	3.1	>10,000	6.3 + 3.1	50.	0.2
	C + S × 25	100.	200.	50 + 25	12.5	0.2
Staphylococcus 12	0 × 25	12.5	6.3	6.3 + 3.1	12.5	>400.
	C × 25	25.	12.5	12.5 + 6.3	12.5	>400.
	S × 25	1.6	>10,000	6.3 + 3.1	12.5	>400.
	C + S × 25	50.	25.	12.5 + 6.3	25.	>400.
Staphylococcus 19	0 × 25	12.5	25.	6.3 + 3.1	12.5	>400.
	C × 25	25.	25.	25 + 12.5	12.5	>400.
	S × 25	3.1	>10,000	3.1 + 1.6	25.	>400.
	C + S × 25	25.	100.	12.5 + 6.3	50.	>400.
Staphylococcus 29	0 × 25	12.5	25.	6.3 + 3.1	12.5	0.2
	C × 25	50.	25.	25 + 12.5	12.5	0.2
	S × 25	6.3	>10,000	12.5 + 6.3	12.5	0.2
	C + S × 25	50.	25.	25 + 12.5	12.5	0.4
Staphylococcus 35	0 × 25	12.5	50.	6.3 + 3.1	12.5	>400.
	C × 25	50.	12.5	25 + 12.5	12.5	>400.
	S × 25	6.3	>10,000	6.3 + 3.1	50.	>400.
	C + S × 25	50.	100.	50 + 25	200.	>400.
Staphylococcus 39	0 × 25	12.5	25.	12.5 + 6.3	12.5	200.
	C × 25	50.	50.	25 + 12.5	50.	100.
	S × 25	6.3	>10,000	12.5 + 6.3	50.	25.
	C + S × 25	100.	200.	50 + 25	50.	100.
Enterococcus 1651	0 × 25	12.5	25.	12.5 + 6.3	50.	1.6
	C × 25	>400.	50.	100 + 50	100.	1.6
	S × 25	6.3	>10,000	25 + 12.5	400.	1.6
	C + S × 25	100.	100.	100 + 50	200.	1.6
Strep. viridans 9	0 × 25	6.3	12.5	3.1 + 1.6	400.	0.01
	C × 25	6.3	12.5	12.5 + 6.3	400.	0.01
	S × 25	0.8	>10,000	6.3 + 3.1	50.†	0.01
	C + S × 25	12.5	12.5	12.5 + 6.3	200.	0.01

*0 = no antibiotic; C = chloramphenicol; S = streptomycin (combination used in constant ratio C; S = 2.1).

†Spare growth in control culture.

Cross-resistance was not demonstrated between chloramphenicol and each of the other 3 antibiotics following the 25 subcultures on the individual agents, except that some of the strains appeared to have increased in resistance to erythromycin 4-fold or more following the exposures to chloramphenicol (Table VI). Also, Staphylococcus 9 and 35 showed an apparent increase in sensitivity to streptomycin following the subcultures on chloramphenicol (Table IV), while the reverse was true of 4 strains (Staph. Nos. 10, 12, and 19 and *Strep. viridans* 9).

Following the subcultures on the mixtures: (1) More than half of the strains exposed to chloramphenicol and streptomycin showed a significant increase in resistance to the individual components of the mixture (Table IV). (2) There was a slight increase in resistance to the components of chloramphenicol-penicillin combination in some of the strains (Table V). (3) All of the staphylococcal strains which had been exposed to the mixture of chloramphenicol with erythromycin increased appreciably in resistance to erythromycin but not to chloramphenicol (Table VI).

TABLE V. EFFECT OF 25 SUBCULTURES ON AGAR CONTAINING PENICILLIN, CHLORAMPHENICOL, OR BOTH ON THE SENSITIVITY OF 9 BACTERIAL STRAINS TO THESE ANTIBIOTICS

STRAIN	NUMBER OF SUBCULTURES*	MINIMUM INHIBITING CONCENTRATION, μG/ML.		
		PENICILLIN (P)	CHLORAMPHENICOL (C)	P + C†
Staphylococcus 10	0 × 25	0.05	6.3	0.01 + 3.1
	P × 25	>400.	3.1	0.01 + 3.1
	C × 25	0.1	25	0.05 + 12.5
	P + C × 25	0.1	25	0.05 + 12.5
Staphylococcus 13	0 × 25	0.05	6.3	0.01 + 3.1
	P × 25	0.4	6.3	0.02 + 6.3
	C × 25	0.1	50.	0.05 + 12.5
	P + C × 25	0.2	25.	0.05 + 12.5
Staphylococcus 14	0 × 25	0.05	12.5	0.02 + 6.3
	P × 25	100.	3.1	0.02 + 6.3
	C × 25	0.05	50.	0.05 + 12.5
	P + C × 25	0.1	25.	0.05 + 12.5
Staphylococcus 27	0 × 25	0.05	12.5	0.02 + 6.3
	P × 25	200.	6.3	0.02 + 6.3
	C × 25	0.1	50.	0.05 + 12.5
	P + C × 25	0.1	25.	0.05 + 12.5
Staphylococcus 29	0 × 25	0.05	12.5	0.02 + 6.3
	P × 25	100.	6.3	0.02 + 6.3
	C × 25	0.05	50.	0.05 + 12.5
	P + C × 25	0.2	100.	0.1 + 25
Strep. viridans 9	0 × 25	0.01	6.3	0.005 + 1.6
	P × 25	0.1	3.1	0.01 + 3.1
	C × 25	0.01	6.3	0.01 + 3.1
	P + C × 25	0.02	3.1	0.01 + 3.1
Strep. viridans 10	0 × 25	0.02	6.3	0.005 + 1.6
	P × 25	0.04	3.1	0.01 + 3.1
	C × 25	0.02	6.3	0.01 + 3.1
	P + C × 25	0.04	3.1	0.01 + 3.1
Enterococcus 5	0 × 25	3.1	12.5	1.6 + 6.3
	P × 25	50.	12.5	3.1 + 12.5
	C × 25	3.1	100.	1.6 + 6.3
	P + C × 25	6.3	25.	3.1 + 12.5
Enterococcus 6	0 × 25	3.1	25.	1.6 + 6.3
	P × 25	25.	12.5	1.6 + 6.3
	C × 25	1.6	>400.	1.6 + 6.3
	P + C × 25	3.1	12.5	1.6 + 6.3

*0 = no antibiotic.
†Constant ratio of P:C maintained at 1:4 for enterococci and at 1:256 for all others.

After the 25 subcultures on chloramphenicol and/or streptomycin the strains neither increased nor decreased in sensitivity to penicillin (3 of the staphylococcal strains were originally highly resistant to penicillin), but in-

creases in resistance to neomycin occurred irregularly and to a slight degree in the strains which had increased in their resistance to streptomycin (Table IV). The strains which had been subcultured on chloramphenicol and/or erythromycin showed no changes in resistance to penicillin (except in Staphylococcus 9), but most of the strains exposed to erythromycin alone increased considerably in resistance to carbomycin, and 3 strains increased 4-fold in resistance to the latter after the 25 subcultures in the mixture (Table VI).

TABLE VI. EFFECT OF 25 SUBCULTURES ON AGAR CONTAINING CHLORAMPHENICOL, ERYTHROMYCIN, OR BOTH ON THE SENSITIVITY OF 9 BACTERIAL STRAINS TO THESE ANTIBIOTICS AND TO CARBOMYCIN AND PENICILLIN

STRAIN	SUBCULTURES*	MINIMUM INHIBITING CONCENTRATION, μG/ML.				
		CHLORAMPHENICOL	ERYTHROMYCIN	CHLORAMPHENICOL + ERYTHROMYCIN	CARBOMYCIN	PENICILLIN
Staphylococcus 9	0 × 25	12.5	0.4	3.1 + 0.2	25.	0.1
	C × 25	25.	0.4	3.1 + 0.2	50.	0.1
	E × 25	12.5	6.3	6.3 + 0.4	200.	0.05
	C + E × 25	25.	12.5	6.3 + 0.4	50.	3.1
Staphylococcus 10	0 × 25	12.5	0.4	3.1 + 0.2	25.	0.1
	C × 25	50.	1.6	12.5 + 0.8	400.	0.1
	E × 25	6.3	3.1	6.3 + 0.4	50.	0.1
	C + E × 25	25.	3.1	12.5 + 0.8	100.	0.1
Staphylococcus 12	0 × 25	12.5	0.4	3.1 + 0.2	25.	800.
	C × 25	25.	0.8	6.3 + 0.4	50.	200.
	E × 25	12.5	3.1	6.3 + 0.4	100.	800.
	C + E × 25	50.	6.3	25 + 1.6	100.	800.
Staphylococcus 19	0 × 25	12.5	0.4	3.1 + 0.2	25.	400.
	C × 25	50.	3.1	12.5 + 0.8	50.	400.
	E × 25	12.5	800.	12.5 + 0.8	>800.	800.
	C + E × 25	12.5	3.1	6.3 + 0.4	50.	400.
Staphylococcus 29	0 × 25	25.	0.4	3.1 + 0.2	25.	0.1
	C × 25	200.	6.3	25 + 1.6	50.	0.1
	E × 25	50.	25.	50 + 3.1	50.	0.1
	C + E × 25	12.5	3.1	12.5 + 0.8	50.	0.1
Enterococcus 3	0 × 25	25.	0.4	3.1 + 0.2	25.	3.1
	C × 25	25.	0.4	3.1 + 0.2	25.	3.1
	E × 25	12.5	50.	6.3 + 0.4	12.5	6.3
	C + E × 25	25.	12.5	12.5 + 0.8	50.	3.1
Enterococcus 6	0 × 25	25.	0.8	3.1 + 0.2	25.	3.1
	C × 25	>400.	0.2	3.1 + 0.2	25.	3.1
	E × 25	12.5	>800.	12.5 + 0.8	>800.	3.1
	C + E × 25	25.	3.1	6.3 + 0.4	25.	3.1
Strep. viridans 9	0 × 25	3.1	0.005	0.2 + 0.01	12.5	0.01
	C × 25	6.3	0.02	0.4 + 0.02	6.3	0.01
	E × 25	6.3	0.1	1.6 + 0.1	>800.	0.01
	C + E × 25	3.1	0.04	0.4 + 0.02	12.5	0.01
Strep. viridans 10	0 × 25	6.3	0.04	0.8 + 0.04	0.4	0.02
	C × 25	6.3	0.04	0.4 + 0.02	1.6	0.02
	E × 25	6.3	0.04	0.8 + 0.04	3.1	0.02
	C + E × 25	6.3	0.04	0.4 + 0.02	1.6	0.02

*0 = no antibiotic; C = chloramphenicol; E = erythromycin; C:E = 16:1.

Miscellaneous Observations.—Many of the strains which had increased in their resistance to one or another of the antibiotics or combinations showed obvious changes in their rate of growth as compared with the growth of the

control cultures which had not been exposed to antibiotics; this was noted both in the presence of subinhibitory concentrations of the antibiotics and occasionally after additional subcultures without antibiotic. Changes in colonial and microscopic morphology were also noted, particularly among the staphylococci during the subcultures on antibiotic media; but this aspect was not studied specifically. The heat resistance of the strains of enterococci was not affected in any of the studies. The hemolytic property of the staphylococcal and enterococcal strains and the effect of the strains of *Strep. viridans* on the blood in the agar medium during growth was not affected by the antibiotics.

Tests for coagulase were done with all strains of staphylococci on several occasions in the course of each of the experiments. All the strains were coagulase positive at the beginning of the studies. During the course of the subcultures some irregularities were noted; these were infrequent and inconstant during the studies with 4 of the pairs of antibiotics but negative tests were frequently noted with some of the strains in the experiments with penicillin and chloramphenicol and with erythromycin and chloramphenicol. The results seemed to vary when plasmas from different donors were used, when the organisms were taken directly from the surfaces of antibiotic-containing media, or when different periods of incubation were used. Since the method employed was not adequately standardized, little can be said beyond the fact that the coagulase-producing property of the staphylococci may have been altered temporarily during the exposures to some antibiotics; this may have been due to residual antibiotic present with the bacterial cells, or to some technical features of the test.

Tests for penicillinase were made on all of the staphylococcal strains at the start of the study and after the twenty-fifth subcultures of each of the 4 series in all of the experiments. The modified Gots method was used.[29] All strains which were initially resistant to penicillin also produced penicillinase and this property persisted in every instance throughout each series of subcultures. Those which were initially sensitive to penicillin did not produce penicillinase either before or after the exposures to the different antibiotics and even after they had become resistant to penicillin following the subcultures in that agent. In some of the tests, when organisms that had originally produced penicillinase were taken directly from the surfaces of plates containing high concentrations of antibiotics, particularly erythromycin, it was not possible to demonstrate the production of penicillinase by this method; the same strains, however, produced the enzyme after a single subculture on antibiotic-free media.

DISCUSSION

From the data presented it is seen that, with the method used, the development of resistance or cross-resistance to any of the antibiotics or pairs of antibiotics studied varied with the antibiotics to which the organisms were exposed and also, to some extent, with the species or strains that were tested. Some of the variations may have been due to the rather crude method employed, which may explain some of the quantitative differences obtained when the same strain and antibiotic were used in different experiments. Also, the difference in the

rate of development of resistance to the same agents in staphylococci and in streptococci may depend, at least in part, on differences in their rates of growth; the latter, and particularly the strains of *Strep. viridans*, grew more slowly and their final growth was sparser than that of the staphylococci. Estimates of the actual numbers of organisms inoculated with each transfer were made on several occasions by colony counts of broth suspensions of the loopful before and after they were inoculated in the culture plates; for the staphylococcal strains each inoculum usually contained between 1 and 10 million viable organisms, for the enterococci about one-tenth, and for the strains of *Strep. viridans* one one-hundredth of that number.

The observations presented are exploratory and represent part of an attempt to uncover the behavior of antibiotics when used in combinations, and also to determine whether variations may be discerned in the reaction of bacteria to different pairs of effective antibiotics. The number of strains used was necessarily small, with emphasis placed on staphylococci to which these studies were particularly oriented. In addition, it was convenient technically to maintain a constant ratio between the concentrations in each pair of antibiotics and the ratio chosen may have been an important factor in some of the results. Strains which were initially sensitive to each component of the mixture were chosen for each combination and the ratio of the concentrations selected depended on the original inhibiting concentrations of the individual agents.

Preliminary studies on strains resistant to one of the components of a mixture have also been carried out and are reported elsewhere.[30] In that study penicillin-resistant staphylococci were repeatedly subcultured in the presence of a constant and high, but noninhibitory concentration of penicillin together with graded concentrations of either streptomycin or erythromycin; the presence of the penicillin did not seem to delay or suppress the development of resistance to the other antibiotics of the mixtures.

The mechanism by which a combination of antibiotics delays or depresses resistance to the pair or to the individual agents is not clear. Nor is the relation of these findings to the problem of synergism and antagonism at all apparent. Some of these problems have been discussed recently by Klein and Schorr,[6] who studied this phenomenon from a different point of view and by different techniques. From a practical point of view, however, the present findings suggest that some delay and depression in antibiotic resistance may result under certain circumstances from exposure of staphylococci, and some streptococci, to any of the 6 pairs of antibiotics studied here, or to the combination of erythromycin with either penicillin or streptomycin, as previously demonstrated,[25] when compared with the resistance that develops in the same strains exposed to the same antibiotics individually.

The present studies have shed no light on one important feature of penicillin-resistant staphylococci, namely, the difference between those isolated directly from patients (whether in relation to penicillin therapy or independently of such treatment), which are invariable penicillinase producers, and strains derived in vitro following exposure to penicillin of originally sensitive staphylococci, which are of equal or greater resistance to penicillin but never-

theless do not produce penicillinase. One possible explanation is that in vivo penicillin-resistant and penicillinase-producing variants are present among the sensitive strains and are permitted to multiply selectively under the impact of the inhibitory or bactericidal action of penicillin which eliminates penicillin-sensitive cells. An alternative and plausible explanation is that penicillinase production in the case of staphylococci requires some constituent in the menstruum which can be supplied by body fluids but not by the usual culture media. Other organisms, however, may acquire the capacity to produce penicillinase in vitro; this was demonstrated by Pollock[31] who found that cells of *Bacillus cereus* reacted to contact with penicillin for one hour at 0° C. by rapidly forming penicillinase when subsequently incubated in a penicillin-free casein hydrolysate medium.

The significance of these findings in terms of the clinical use of antibiotic combinations, particularly in the prolonged treatment of staphylococcal or enterococcal infections or of subacute bacterial endocarditis, must await studies in which the sensitivities of strains persisting after therapy with these agents, alone or in pairs, are studied and compared. Clinical usage, however, offers conditions which are not duplicated in the present vitro experiments, since, for the most part, antibiotics are given either in maximum tolerated doses or in doses which can be varied only within very narrow limits as compared to those employed here. Moreover, clinical data of this type and under reasonably controlled conditions are very difficult to obtain.

SUMMARY AND CONCLUSIONS

Strains of Staphylococcus, Enterococcus and *Streptococcus viridans* were subcultured 25 times on blood agar containing increasing concentrations of penicillin, streptomycin, Terramycin, erythromycin, or chloramphenicol, either individually or in pairs; they were then tested for sensitivity to each of the individual or pairs of antibiotics to which they had been exposed and the results compared with those of similar tests done on the parent strains after a similar number of subcultures in parallel on antibiotic-free media. Tests for sensitivity to neomycin, Aureomycin, and carbomycin were also done on all strains in experiments in which streptomycin, Terramycin and erythromycin, respectively, had been used.

The results, which are summarized in Table VII, extend observations with combinations of erythromycin and penicillin or streptomycin[25]; they show that the development of resistance by staphylococci and certain streptococci following repeated subcultures in penicillin, streptomycin, erythromycin, Terramycin, or chloramphenicol may be depressed and delayed but not prevented by subculturing the organisms in pairs of these antibiotics in any of the 8 combinations studied. They also show that the rate and degree of resistance which developed against the individual antibiotics to which the organisms had been exposed varied with the antibiotics and sometimes with the strains.

Resistance developed most regularly and rapidly to streptomycin, and only slightly less so to erythromycin and penicillin, except for some of the strep-

tococcal strains which showed only small increases in resistance to penicillin. Resistance to Terramycin developed quite regularly but usually to a lower degree than to the previous 3 agents, while the exposures to chloramphenicol resulted in only slight or no increases in resistance to that agent. Subcultures in pairs of antibiotics resulted in increased resistance to those mixtures, but

TABLE VII. SUMMARY OF CHANGES IN RESISTANCE OBSERVED FOLLOWING 25 SUBCULTURES ON BLOOD AGAR CONTAINING ANTIBIOTICS, SINGLY OR IN PAIRS

ANTIBIOTICS WITH WHICH ORGANISMS WERE SUBCULTURED	ANTIBIOTICS TO WHICH RESISTANCE DEVELOPED				
	PENICILLIN	STREPTOMYCIN	PENICILLIN + STREPTOMYCIN	NEOMYCIN	
Penicillin	++++(+[a])	0, –	0	– (0[a])	
Streptomycin	0	++++	0(+[a])	+, ++	
Penicillin + streptomycin	++(0[a], +[b])	+++(++[a], 0[b])	++(+[a, b])	+	
	PENICILLIN	TERRAMYCIN	PENICILLIN + TERRAMYCIN	AUREOMYCIN	
Penicillin	++++(+[a])	0	0	0(–[a, b])	
Terramycin	0	+++(+[a, b])	0	++(+[a], 0[b])	
Penicillin + Terramycin	+(0[a, b])	+(0[b])	++(+[a, b])	+(0[a, b])	
	ERYTHROMYCIN	TERRAMYCIN	ERYTHROMYCIN + TERRAMYCIN	CARBOMYCIN	AUREOMYCIN
Erythromycin	++++	0, –	0(++[b])	+++	0, –
Terramycin	0	++(+[a])	0	0	+
Erythromycin + Terramycin	+++	++(+[a], 0[b])	++	++	+
	CHLORAMPHENICOL	STREPTOMYCIN	CHLORAMPHENICOL + STREPTOMYCIN	NEOMYCIN	PENICILLIN
Chloramphenicol	0, +(+++[a])	0, –	+	0	0
Streptomycin	–	++++	0	+	0
Chloramphenicol + streptomycin	+	+	+	0, +	0
	CHLORAMPHENICOL	PENICILLIN	CHLORAMPHENICOL + PENICILLIN		
Chloramphenicol	+(0[b])	0	0		
Penicillin	0	++++(+[a, b])	0		
Chloramphenicol + penicillin	0, +	0, +	0, +		
	CHLORAMPHENICOL	ERYTHROMYCIN	CHLORAMPHENICOL + STREPTOMYCIN	CARBOMYCIN	PENICILLIN
Chloramphenicol	+(0[b])	0, +	0, +	+	0
Erythromycin	0	+++	0, +	++	0
Chloramphenicol + erythromycin	0	+, ++	0, +	0, +	0

Explanation of table: Changes are compared with inhibiting concentrations of control strain subcultured on antibiotic-free media and are expressed as follows: 0 = no change (i.e., same, twice, or one-half); + = 4- or 8-fold increase in resistance; ++ = 16- or 32-fold increase in resistance; +++ = 64- or 128-fold increase in resistance; ++++ = 256-fold or greater increase in resistance; – = more sensitive (requiring one-fourth or less of original inhibiting concentration).

For convenience, when the results varied, they were indicated in some instances as rough averages and in others as a range, e.g., "0, +." When the results obtained with the streptococcal strains differed significantly from those obtained with staphylococci, they are shown in parentheses, results with the enterococci being indicated by the superscript *a* and those with *Str. viridans* by superscript *b*.

usually of lower degree than those observed with the individual components. When the increases in resistance to the homologous individual agents differed appreciably, the resistance to the pair corresponded more clearly to that of the component which produced the smaller change. Increases in resistance to the individual antibiotics of a mixture usually resulted from exposure to the combination but these were of lower magnitude than the increases which developed to the homologous individual components of the combination of the same antibiotics.

Cross-resistance between the separate components of the pairs of antibiotics used in this study was not observed, except for increased resistance of some strains to erythromycin after exposures to chloramphenicol. However, in some strains, the exposures to one of the agents resulted in an apparent increase in sensitivity to the other. Some cross-resistance to neomycin was observed in many of the strains which had increased in resistance to streptomycin; moderate increases in resistance to Aureomycin were noted in most of the strains which had been exposed to Terramycin while almost all of the strains exposed to erythromycin developed cross-resistance to carbomycin.

Acknowledgments.—We are indebted to Mr. Frank P. Schley of Parke, Davis & Company for the chloramphenicol, to Dr. Gladys L. Hobby of Chas. Pfizer & Co., for the Terramycin and carbomycin (Magnamycin), to Dr. J. W. Smith of Lilly Research Laboratories for the erythromycin, to Dr. Stanton M. Hardy of the Lederle Laboratories for the Aureomycin, and to Dr. Earl L. Burbidge of The Upjohn Company for the neomycin.

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SEROLOGIC DIAGNOSIS WITH RESPECT TO HISTOPLASMOSIS, COCCIDIOIDOMYCOSIS, AND BLASTOMYCOSIS AND THE PROBLEM OF CROSS REACTIONS

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THE definitive diagnosis of infection with a pathogenic fungus, at the present time, requires either the isolation of the agent or the demonstration of its presence in infected tissues. These are time-consuming and, for many reasons, frequently unsatisfactory procedures. Hence, considerable effort has been directed to the development of serologic techniques. Complement fixation, precipitin and collodion agglutination tests are currently employed, but their interpretation is often difficult. In fact, serologic reactions are sometimes completely inconsistent with cultural and clinical findings. This is because of the variations in antibody level found with different types of disease and the occurrence of cross-reactivity between the various mycotic antigens. Seemingly contradictory results, however, become more understandable if the protean nature of these infections and the antigenic relationship of the agents which cause them are considered in their evaluation.

The purpose of this report is to review certain antibody patterns which appear to be associated with several clinical types of histoplasmosis; to call attention to the cross-reactivity of mycotic antigens; and, finally, to describe cases of proved mycotic disease with negative serology. In each instance the serologic configurations will be illustrated by examples drawn from cases studied in this laboratory.

MATERIALS AND METHODS

A. *Antigens.*—

1. The coccidioidin,* histoplasmin,† and blastomycin‡ used were crude filtrates of liquid synthetic media in which mycelial strains of *Coccidioides immitis, Histoplasma capsulatum,* and *Blastomyces dermatitidis* were cultured over a period of months.

2. Yeast phase antigens of *H. capsulatum* and *B. dermatitidis* were prepared essentially as described in a previous report,[1] except that the saline extracts of the mechanically ground organisms were made from cultures grown on brain heart infusion agar (Difco) instead of the cystine agar used initially. Extracts have also been lyophilized in 1 ml. aliquots and reconstituted on the day of use in order to prevent deterioration of antigenic components and development of anticomplementary activity which occurred when antigens were stored for prolonged periods at 4° C.

B. *Serologic Tests.*—

1. *Complement Fixation (CF)*: As described in earlier reports,[1-3] the technique employed in this laboratory utilizes three 50 per cent units of complement. An eighteen-

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Received for publication, July 7, 1953.

*Lots 47 and 47-54 supplied by Dr. C. E. Smith, University of California.

†Lots H-40 and H-42 supplied by Dr. Arden Howell, Jr., Federal Security Agency, and Lot H-15 supplied by Parke, Davis & Company.

‡Lots B-7 and B-9 supplied by Dr. F. W. Cross, Federal Security Agency.

hour incubation period at 4° C. is allowed for fixation, and an additional period of thirty minutes at 37° C. for the hemolytic system. Tubes containing unlysed cells are centrifuged at 2,500 r.p.m. for five minutes, after which the percentage of hemolysis in the supernate is compared with quantitative hemolytic standards. Serum dilutions in which there is 0 to 50 per cent hemolysis are considered positive.

Coccidioidin and the yeast phase antigens of *H. capsulatum* and *B. dermatitidis* were used routinely in this technique.

2. *Collodion Agglutination (CA):* This test, in which collodion particles sensitized with histoplasmin served as antigen, was described in an earlier report.[4] Originally, the agglutination end point was expressed by the highest serum dilution in which reaction was noted. However, when additional use of the test revealed that the intensity of a reaction was as significant as the serum titer, the procedure was adopted of adding the "pluses" in a serial series of serum dilutions. Thus, a serum with a 4+ reaction at a dilution of 1:5, 4+ reaction at 1:10, 3+ at 1:20, 1+ at 1:40, etc., was assigned a CA value equal to 4 + 4 + 3 + 1, or 12. CA values of 5 to 9 were considered equivocal, while values above 10 were regarded as a positive result.

Histoplasmin was used as the sensitizing agent, except in certain cross-reaction studies in which blastomycin was utilized as well. Coccidioidin was uniformly unreactive in this test.

3. *Precipitin:* These tests, employed only in cross-reaction studies, were carried out with the three mycelial filtrate antigens. The method described by Smith and associates[5] was adopted. This consists of layering 0.2 ml. quantities of undiluted, 1:10, and 1:40 dilutions of antigen over 0.2 ml. amounts of undiluted serum. The tubes are incubated for five days at 37° C. The presence of a ring at the serum-antigen interface or the formation of a precipitate "button" at the bottom of the tube is considered a positive reaction. Both sera and antigens are treated with Merthiolate (1:10,000) in order to diminish bacterial growth during incubation.

RESULTS

I. Antibody Patterns in Histoplasmosis.—

Thirty-seven cases of histoplasmosis, verified by isolation of the organism, were systematically studied for antibody. These infections were in residents of nonendemic as well as endemic areas and included patients in the early primary, moderately advanced, and chronic, generalized stages. Although there was considerable variation between individual responses, four distinct patterns of antibody response could be recognized in these cases. These four patterns, discussed below, are exemplified by the findings in selected cases on which additional information is supplied in Table I.

1. *The Serologic Pattern in Primary, Pulmonary, Self-Limiting Infections:* These infections were characterized by an acute onset, spiking fever, malaise, headache, chest pains, and pneumonitis of varying degree. Chest x-rays revealed a diffuse, mottled infiltration throughout both lungs. Recovery was without apparent complication and occurred in from two to six months. Serial serum specimens were obtained from ten cases of this type and study of these sera revealed that CF antibody appeared very early, with maximum titers ranging between 1:80 and 1:2,560. The peak titers usually were found in the specimens taken within the first six weeks after onset. However, this high level was present for only a short period, and was followed by a decline, so that by the fourth month the antibody titer in many cases was 1:20 or less. A typical CF pattern is shown in Fig. 1, Case 1.

TABLE I. ADDITIONAL INFORMATION ON CASES OF HUMAN HISTOPLASMOSIS USED TO ILLUSTRATE CF ANTIBODY PATTERNS ENCOUNTERED IN A SERIES OF 37 PERSONS WITH THIS DISEASE

CASE	AGE	SEX	CLINICAL DIAGNOSIS	SOURCE OF H. CAPSULATUM ISOLATE	DURATION OF DISEASE BEFORE SEROLOGIC STUDY	DURATION OF SEROLOGIC STUDY	REMARKS
L.E.B.	37	M	Primary pulmonary histoplasmosis	1) Blood 2) Sputum	6 weeks	9 months	See Case 1, Fig. 1
A.C.	44	M	Progressive disseminated histoplasmosis	1) Skin 2) Epiglottis 3) Sputum	5 mo. after onset current episode (Similar episodes during past 4½ years)	16 months (still under observation)	1) See Case 2, Fig. 1 2) Reported by Ellis, et al.: J. Anti. & Chemo. 2: 347-350, July, 1952
R.G.	37	M	1) Generalized histoplasmosis 2) Endocrinopathy, type undetermined	1) Blood 2) Bone marrow 3) Tongue lesion 4) Widespread at autopsy	3 months	9 months	See Case 3, Fig. 1
E.T.	32	F	Primary histoplasmosis —mild	Sputum	5 weeks	12 months	See Text—I (4) Histoplasma infections without detectable CF antibody
R.L.	41	M	Pulmonary histoplasmosis —advanced	Sputum	2-6 years	14 months	do
L.J.	22	F	Cutaneous histoplasmosis	Leg lesions	1 year	36 months	do

Sera from this group of patients cross reacted to an equal degree with a similar type of antigen prepared from *B. dermatitidis*. On the other hand, all were essentially negative with coccidioidin.

2. *The Serologic Pattern in Chronic Generalized Cases:* Only three cases of this nature were available for study. Although the date of onset could not be definitely established in two of them, these patients had experienced recurrent episodes of "pneumonia" within the past two to five years. Chronic malaise, low-grade fever, loss of weight, and mucomembranous lesions were the predominant symptoms. Roentgenograms also revealed varying degrees of hilar infiltration. *H. capsulatum* was repeatedly cultured from the sputa, blood, and cutaneous lesions. One of these patients has died, a second has an unfavorable prognosis, and the third is reported to be clinically improved following therapy.

As might be anticipated if one assumes continued presence of the antigen in the patient, each of these three individuals maintained high CF titers for prolonged periods. Case 2, Fig. 1, illustrates the CF pattern in the one patient who has apparently recovered. The clinical improvement is accompanied

by a gradual decline in titer from 1:320 to 1:40. Unfortunately, early sera were not available on the other two cases. However, during the period of study (at least six months) CF antibody levels were maintained at 1:160 and 1:2,560, respectively.

In contrast to the self-limiting cases of histoplasmosis, whose sera consistently crossed with blastomyces antigens, these advanced cases were erratic in this regard. All sera from two of the patients fixed complement to the same titer with blastomyces antigen, whereas in those from the third, reactions with this antigen were never encountered. No serum from this group of patients was positive with coccidioidin.

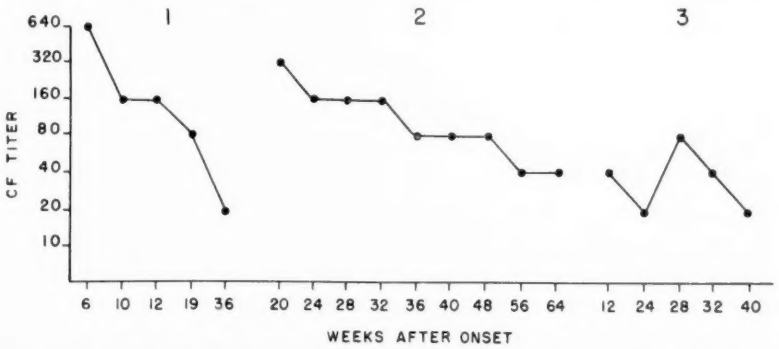


Fig. 1.—Complement fixation antibody patterns in patients with histoplasmosis as demonstrated with yeast phase antigen. *1*, Primary pulmonary histoplasmosis—recovered. *2*, Pulmonary histoplasmosis plus mucocutaneous lesions—apparently recovered. *3*, Generalized histoplasmosis plus mucocutaneous lesions plus Hodgkin's disease—fatal.

3. *The Serologic Pattern in Advanced, Generalized Histoplasmosis Associated With Severe Endocrinopathy:* These ten cases, in whom the fungus had parasitized nearly every tissue, were complicated by either Addison's or Hodgkin's disease or severe endocrinopathy of undetermined origin. In spite of the extreme dissemination of *H. capsulatum*, there was no history of earlier pneumonitic infection and the lung fields in three cases were clear. Although a pronounced listlessness, low-grade fever, and progressive decline in weight had been apparent over several years, all of these patients sought medical assistance because of necrotic ulcers in the throat or mouth or because of a persistent diarrhea. In such cases, the disease was usually well advanced prior to diagnosis, and the earliest sera available for study were not taken until four to six months before death. Eight of the ten patients having this type of advanced, apparently irreversible stage of the disease have already died. In all of these cases there was a consistently low CF titer with levels ranging from less than 1:5 to 1:80. Case 3, Fig. 1, is illustrative of the highest reactions noted in this group. More often, antibody detectable by the CF test was completely absent.

In spite of the fact that reactivity with homologous antigen was very low, low cross reactions were also observed with blastomyces. None was noted with coccidioidin.

4. *Histoplasma Infection Without Detectable CF Antibody:* The final group was composed of cases from whom *H. capsulatum* was isolated but in

whom antibody response as determined by the CF test was negative or negligible. There were fourteen cases in this group and they showed a considerable variation in their clinical picture. On the other hand, early serum specimens from eight very mild pulmonary cases in this group were positive in the CA test. Four cases whose only apparent lesions were cutaneous also reacted in this fashion. The sera of the other two patients, although their infections were of two and fourteen years' duration, respectively, have never been positive in either test.

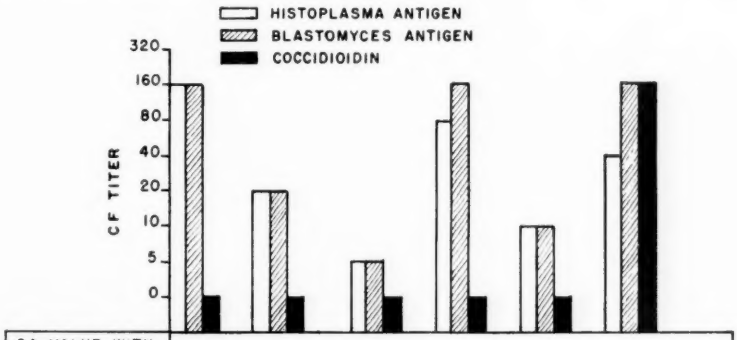
II. Cross Reactions Obtained With Histoplasma, Blastomyces and Coccidioidin Antigens With Sera From Proved Cases of Systemic Mycoses.—

The CF patterns described in the preceding sections indicate that antibody levels in histoplasmosis may vary considerably. In view of the many clinical types of the infection that have been described, this is not surprising.[6-9] However, it is pertinent to note that (as shown in Case 3, Fig. 1) CF titers do not always reflect the severity of the disease. This is in contrast to the CF antibody levels found in extensively disseminated coccidioidomycosis and blastomycosis in which titers increase with progression of infection and remain elevated throughout the terminal stages.[5, 10] Unfortunately, the diagnostician is not always able to rely upon reactions with homologous antigens in establishing the etiology of these fungal infections. Not only do sera from cases from which a specific fungus is isolated react with heterologous antigens, but these reactions will sometimes be higher and, in some cases, the only ones obtained. Furthermore, cross reactivity appears to be associated with certain antigens to a greater extent than others.[11-13] Examples of such instances, using cases in which diagnosis was confirmed by isolation of the organism, are described in the following section. It should be emphasized that these reactions were selected because of the frequency with which they occurred, rather than because they were aberrant and occasionally observed.

1. *Serologic Cross Reactions in Histoplasmosis Cases:* In our experience, the serum from a patient with histoplasmosis may fix complement with blastomyces antigen at essentially the same titer as the homologous antigen. These cross reactions are encountered at all stages of the disease. Cases 4 and 5, Fig. 2, illustrate these findings. Although Case 4 is a primary infection of approximately six weeks' duration and Case 5 is a patient in the tenth and terminal month of disease, the cross reactions obtained with blastomyces antigen equal the titers of the homologous test substance. It is worthy of mention that out of 345 suspected but *not proved* cases of mycotic disease equal titers of 1:10 to 1:320 were obtained with histoplasma and blastomyces antigens in 242 instances, or 75 per cent. On the other hand, sera with high histoplasma CF titers of 1:160 to 1:2,560 never reacted with coccidioidin to a titer greater than 1:20. Even this low level was not encountered in sera from patients with histoplasmosis having titers of 1:80 or below.

2. *Serologic Cross Reactions in Blastomycosis Cases:* The serologic reactions with homologous antigen in our series of 65 culturally and clinically verified cases of blastomycosis generally corroborated those reported by Mar-

tin,[10] who noted that CF antibody levels obtained with homologous antigen were low or absent in cutaneous or otherwise localized infections and high in the disseminated disease. As would be anticipated from the data described in the preceding section, however, we also found that histoplasma antigens cross reacted with sera from all stages of blastomycosis. For example, the serum shown in Case 6, Fig. 2, was an initial specimen submitted two months after the appearance of a small mycotic lesion on the leg, whereas Case 7 is represented by a single, terminal specimen taken ante mortem and approximately ten months after onset. It will be seen that reactions with both antigens are equal in each case. Coccidioidin, on the other hand, failed to react with sera from any of the 65 patients.



CA VALUE WITH HISTOPLASMIN	22	0	22	0	16	20
ORGANISM ISOLATED	H.C.	H.C.	B.D.	B.D.	C.I.	C.I.
RESIDENCE	IND.	TEX.	PENNA.	VA.	TRANSIENT	VA.(VAH)
CASE NO.	4	5	6	7	8	9

Fig. 2.—Serologic reactions with histoplasma, blastomyces, and coccidioidin antigens and sera from human histoplasmosis, blastomycosis, and coccidioidomycosis infections.

3. *Serologic Cross Reactions in Coccidioidomycosis Cases:* Because of the extreme infectivity of *C. immitis,* bacteriologists working in areas where this disease is endemic are loathe to attempt isolation; and considerable reliance is placed on the demonstration of CF antibodies or precipitins in the patient's serum. Thus, it is essential that the cross reactions which can occur with such sera be clearly understood. As in blastomycosis, the degree of coccidioidomycotic involvement is reflected by the CF antibody level. *Titers greater than 1:40 are always suggestive of dissemination, with the prognosis becoming poorer as the level increases.* On the other hand, specific CF antibody can be low or completely absent throughout the entire course of a mild infection.[5] Unfortunately, it is in these latter cases that marked cross reactions with histoplasma and blastomyces antigens are more frequently obtained. Case 8, Fig. 2, was a patient on the eastern seaboard with a mild pneumonitic infection in which CF antibody was not detectable by coccidioidin. However, there was sufficient reaction with both types of histoplasma antigen to warrant attempts

to isolate a fungus. As a result, *C. immitis* was cultured from the sputum on three occasions, although the serologic response to both histoplasma and blastomyces antigen was greater than to coccidioidin. While not the general rule, sera from severe and rapidly disseminating coccidioidomycosis with high CF antibody levels to coccidioidin may also occasionally cross react. This is illustrated by Case 9, Fig. 2.

To emphasize further that cross reactions occur frequently in sera from patients with coccidioidomycosis, the CF titers with coccidioidin and histoplasma antigens on 77 sera from presumptive coccidioidal infections collected in an endemic area are tabulated in Table II.* Thirty-one (40.3 per cent) specimens reacted to equal or higher titers with histoplasma antigen, although the coccidioidin CF levels ranged from negative to greater than 1:80.

TABLE II. CROSS REACTIONS OBTAINED WITH HISTOPLASMA YEAST PHASE ANTIGEN IN 77 SERA FROM EARLY INFECTIONS OF PRESUMPTIVE COCCIDIOIDOMYCOSIS

NO. SERA TESTED	CF TITER WITH COCCIDIOIDIN	NO. SERA WITH CF TITER TO HISTOPLASMA						
		0	1:5	1:10	1:20	1:40	1:80	1:160
16	Neg.		6	10				
9	1:5	5		2	1		1	
17	1:10	10	2	5				
19	1:20	9	3	2	5			
8	1:40	4		1	2	1		
4	1:80	2		1		1		
4	>1:80	3			1			

III. The Relationship of Antigen Type and Test Procedure to Cross Reactions.—

It has been reported by Salvin[14] that extensive cross reactions seldom, if ever, are encountered when whole yeast cells of *H. capsulatum* are employed as antigen. Our own experience has not corroborated these observations. The sera from ten cases each of coccidioidomycosis and blastomycosis in varying stages of illness were selected at random and simultaneously tested by complement fixation with four different antigenic preparations. The results of this experiment are shown in Table III. Four of the ten sera from blastomycosis (Numbers 1, 2, 3, and 8) and each of the ten specimens from coccidioidomycosis reacted with the whole cell antigen to titers that approximately equaled or exceeded those obtained with the antigens of the homologous agents.

The collodion agglutination test, utilizing collodion particles coated with histoplasmin as antigen, has been used routinely in our laboratory. Cross reactions also occurred with this procedure, although it was markedly positive in early sera from mild, pulmonary cases of histoplasmosis as well as in those with cutaneous lesions in which CF antibody detectable by the yeast phase antigen never developed. These are exemplified in Cases 6, 8, and 9, Fig. 2, and in the 25 additional sera from presumptive cases of coccidioidomycosis

*In early 1948, we joined a collaborative study in coccidioidomycosis with Dr. C. E. Smith, University of California, and the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board. The 102 specimens listed in Tables I and III were among those included in this study and were from cases classified as primary coccidioidal infections with and without erythema nodosum. Complete results of the investigation will be presented in a joint report.

TABLE III. COMPARISON OF CF TITERS IN SERA FROM HUMAN CASES OF BLASTOMYCOSIS AND COCCIDIOIDOMYCOSIS

SERUM NO.	ORGANISM ISOLATED	HISTOPLASMA WHOLE YEAST CELL ANTIGEN	HISTOPLASMA EXTRACT YP* ANTIGEN	BLASTOMYCES EXTRACT YP* ANTIGEN	COCCIDIOIDIN
	Blastomyces dermatitidis				
1		*1:10*	Neg.	Neg.	Neg.
2		*1:5*	Neg.	*1:10*	Neg.
3		*1:40*	1:10	*1:10*	Neg.
4		Neg.	Neg.	*1:20*	Neg.
5		Neg.	Neg.	*1:40*	Neg.
6		Neg.	1:10	*1:80*	Neg.
7		Neg.	1:5	*1:160*	Neg.
8		*1:160*	1:20	*1:160*	Neg.
9		1:20	1:10	*1:160*	Neg.
10		1:5	1:10	1:160	Neg.
	Coccidioides immitis				
1		1:20	1:10	1:40	*Neg.*
2		1:20	1:20	1:20	*Neg.*
3		1:5	Neg.	1:10	*1:5*
4		1:10	Neg.	Neg.	*1:10*
5		1:10	1:5	Neg.	*1:10*
6		1:10	Neg.	Neg.	*1:20*
7		1:20	Neg.	Neg.	*1:40*
8		1:20	1:5	Neg.	*1:40*
9		1:40	1:20	1:10	*1:40*
10		1:40	1:40	1:40	*1:80*

*YP—Yeast Phase.

in Table IV. Although elevated coccidioidin CF titers of 1:80 to 1:640 indicated that the latter specimens were from moderately to severely disseminated infections, significantly high CA values of 10 to 27 were obtained with collodion particles sensitized with histoplasmin.

The precipitin test was not used routinely in these studies. Nevertheless, the cross reactions described above were encountered with this technique also. In addition, coccidioidin, which appeared to be relatively specific for the CF antibody of *C. immitis,* in this method cross reacted with sera from early cases of histoplasmosis. Smith[13] has also reported cross reactions of this nature.

DISCUSSION

Although the test procedures and antigens currently used are still in the developmental stage, the data presented above and the findings of others indicate that in the mycotic diseases one is dealing with infections in which the antibody pattern varies not only with the course of the disease but with the causative agent. This situation is further complicated by the fact that it is

TABLE IV. COLLODION AGGLUTINATION VALUES WITH HISTOPLASMA IN SERA FROM 25 PRESUMPTIVE CASES OF COCCIDIOIDOMYCOSIS

NO. SERA	COCCIDIOIDIN CF TITER	CA NUMERICAL VALUES WITH HISTOPLASMIN
13	1:80	10 to 16
7	1:160	13 to 18
3	1:320	18 to 22
2	1:640	18 and 27

almost impossible to differentiate these infections clinically, especially in the early stages of the pulmonary type. Furthermore, the transfer of military personnel into and out of states where certain of these mycoses have long been prevalent has resulted in the finding of an increased number of proved cases of coccidioidomycosis, histoplasmosis, and blastomycosis in areas which are not considered endemic foci. In view of these circumstances, it is particularly unfortunate that the mycotic antigens currently employed cross react to a marked degree. On the other hand, certain general principles appear to be applicable. We have attempted to summarize the antibody (cross as well as homologous) patterns of these three systemic mycoses in Table V, using our own data as well as others.

TABLE V. SUMMARY OF HOMOLOGOUS AND CROSS REACTION ANTIBODY PATTERNS OBTAINED IN SERA FROM HUMAN CASES OF HISTOPLASMOSIS, BLASTOMYCOSIS, AND COCCIDIOIDOMYCOSIS

		CF TITER WITH			CA	PRECIPITIN	
DISEASE	CLINICAL STATUS	HISTOPLASMA (YEAST PHASE)	BLASTOMYCES (YEAST PHASE)	COCCIDIOIDIN	HISTOPLASMIN	HISTOPLASMIN	COCCIDIOIDIN
Histoplasmosis	Early, pulmonary (self-limiting)	0-1:2,560	0-1:2,560	0-1:20	Postive	Postive	Positive or negative
	Cutaneous	0-1:20	0-1:20	Negative	Postive	Positive	Unknown
	Generalized, advanced (plus severe endocrinopathy)	0-1:40	0-1:40	Negative	Postive or negative	Usually negative	Unknown
Blastomycosis	Cutaneous or other localized foci	0-1:20	0-1:20	Negative	Positive	Positive	Unknown
	Generalized, systemic	0-1:160	1:40-1:320	Negative	Negative	Negative	Unknown
Coccidioidomycosis	Early, primary or other localized foci	0-1:40	0-1:40	0-1:40	Positive or negative	Positive or negative	Positive
	Disseminated	0-1:160	0-1:320	1:40-1:1,280	Positive or negative	Usually negative	Usually negative

1. In patients with primary, acute pulmonary histoplasmosis CF antibodies usually develop rapidly after onset. The elevated titers are transitory in the milder, self-limiting cases but may persist for very long periods in more severe infections of extended duration. Conversely, CF titers are low or absent in the extensively disseminated, usually fatal, cases that are associated with endocrinopathy. They are also negligible in the sera of patients whose only apparent lesions are in cutaneous or mucocutaneous tissues.

Sera from patients with histoplasmosis rarely cross react with an antigen from *C. immitis* in the CF test. When such cross reactions do occur, they are low (1:20) and are invariably accompanied by much higher homologous levels, as 1:160 to 1:2,560. However, sera from all types of histoplasmosis may cross react completely with antigens of *B. dermatitidis*.

2. In blastomycosis, the CF level increases with progression of the disease and remains elevated throughout the terminal stages. On the other hand, antibodies are low or absent in infections limited to cutaneous or other focalized lesions. Sera from patients with blastomycosis frequently react to equal CF titers with antigens of *H. capsulatum*, but are uniformly negative with coccidioidin.

3. The most widespread clinical illness of this group, coccidioidomycosis, also requires the most care in the interpretation of serologic tests. As in blastomycosis, CF titers may be low or absent in self-limited or focalized infections, or as the disease disseminates they may reach levels of 1:40 to 1:1,280. Cross reactions with histoplasma and blastomyces antigens in the latter cases rarely approach the homologous level and often are absent in those of longer duration. On the other hand, sera from the more prevalent, milder types of coccidioidomycosis frequently react to much higher CF titers with these heterologous antigens than with coccidioidin. In the complete absence of detectable homologous CF antibody and low (1:5 to 1:40) titers with histoplasma and/or blastomyces antigens, coccidioidomycosis should still be suspected. It is notable that in our laboratory thirty-five cases of coccidioidomycosis, subsequently confirmed by the isolation of *C. immitis*, were first detected by this pattern with heterologous antigens instead of significant reactions with the antigen of the etiologic agent. It is, therefore, obvious that all three antigens should be employed in the CF test.

At the present time there appears to be no serologic method of choice. It is often possible not only to detect infections, but to follow the course of illness in coccidioidomycosis and blastomycosis and certain types of histoplasmosis by serial CF antibody titration. On the other hand, precipitin and collodion agglutination tests are sometimes positive in milder cases in which CF antibody is never developed.[5, 15] These latter tests, however, not only become negative in proved cases of longer duration, but they are sometimes positive in earlier ones, regardless of whether coccidioidin, histoplasmin, or blastomycin is employed. For these reasons, we use both types of tests in our laboratory.

The type of antigen used, whether cellular or soluble, apparently is of very little importance. While some antigenic preparations may be more reactive than others, cross reactions were demonstrated with all of the crude substances currently employed. Of these three mycotic agents, antigens prepared from *H. capsulatum* appeared to be the most reactive with sera from other mycotic diseases. In fact, cross reactions have been so extensive with histoplasmin in the CA test that we now interpret positive findings as presumptive evidence that an infection is *mycotic* in origin rather than as indicative of invasion with *H. capsulatum* per se. While CF titers of 1:10 to 1:80 with histoplasma yeast phase antigen are interpreted in a similar manner, there is considerable evidence to indicate that levels of 1:160 to 1:2,560 are found only in active infections with this agent.

In conclusion, it should be stressed that the absence of detectable antibody does not rule out infection with pathogenic fungus species. The isolation of these microorganisms from cases with extensive clinical findings and with

essentially negative serology is not infrequent. Indeed, twelve of our thirty-seven cases of histoplasmosis were in this category and, as pointed out in the preceding sections, some were of considerable duration. Thus, while serologic tests have provided a means for detecting many cases which might otherwise have been overlooked, it is evident that isolation attempts still constitute an important part in the definitive laboratory diagnosis of the systemic mycoses.

SUMMARY

The serologic reactions in a series of patients with proved mycotic disease have been described. Certain of these appear to form a consistent pattern that can be used for diagnosis and prognosis. The available antigens are not specific, and the most troublesome types of cross reactivity are discussed.

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LABORATORY METHODS

A NEW METHOD, USING RADIOIRON, FOR DETERMINING THE IRON-BINDING CAPACITY OF HUMAN SERUM

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INTRODUCTION

THE presence of iron in human serum was originally described by Barkan.[1] Later investigations of iron metabolism and ferrokinetics strongly suggested that the iron was transported in combination with a serum protein, and this iron-binding protein was eventually isolated by Surgenor et al.[2] and electrophoretically identified as B_1 globulin, present in Fraction IV-7 of Cohn.[3] In the meantime, other studies by Holmberg and Laurell[4] had implied that the serum was unsaturated with respect to its iron content, and that further increments of ionic iron could be given intravenously before serum saturation was reached and subsequent toxicity symptoms developed. Schade,[5] independently, was able to demonstrate, in the course of bacteriological investigations, the in vitro ability of iron-binding protein to combine with further amounts of iron, and to show that the iron-protein complex produced a color the intensity of which deepened as additional amounts of iron were combined to the protein until saturation was reached.[6]

Numerous conceptual theories have evolved regarding the iron-binding capacity of serum, and many studies have been made regarding its magnitude in health and disease. The measurement techniques which have most commonly been used have been spectrophotometric, and have been based upon Schade's demonstration that stepwise additions of ionic iron to the serum in vitro produced progressive increases in the color of the iron-protein complex, until the protein became saturated. At the saturation point there was usually a sharp break in the curve of color intensity, following which excess ionic iron was not bound to protein, and no further color developed.

One of the problems which immediately arose with the use of the spectrophotometric technique was the optimal wave length of the filter to be used. Surgenor's group showed that the absorption curve of the iron-protein complex had a wide band ranging from 420 to 530 mμ, with maximum absorption at 465 mμ. However, the Rath and Finch[7] method for measuring iron-binding capacity used an absorption filter of 525 mμ to avoid "color interference by

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The technical assistance of Melba Robson and Pearl Avery is gratefully acknowledged.
Received for publication, Aug. 6, 1953.
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**Work, in part, performed during tenure of a Damon Runyon Senior Clinical Research Fellowship.

other serum substances which absorb light at the shorter wave length." Cartwright and Wintrobe,[8] in an essentially similar method, felt that less interference from hemoglobin and bile pigments was encountered with a filter of 490 mμ.

In addition to the question of the optimal wave length for the measurement filter, the spectrophotometric determination of serum iron-binding capacity has other, more significant, disadvantages. The method cannot be used with lipemic or icteric serum, or with serum older than twenty-four hours. The measurement is limited to an accuracy of 50 gamma per cent, the size of the individual additions of iron. Moreover, experience in this laboratory has shown that, despite careful laboratory technique with the modified spectrophotometric method, the points obtained for the color curve do not always produce a sharp break and that the graphical demarcation of the saturation point is often difficult and occasionally impossible.

The availability of radioiron and the elegance of quantitative determinations using Geiger-Müller or scintillation counting suggested that radioiron might be used in the determination of the serum iron-binding capacity. The proposed method would add to serum a quantity of radioiron of known specific activity in an amount greater than that required to saturate the protein, allow the radioiron to combine with the protein, separate the iron-saturated protein from the solution, measure the excess of unbound radioiron, and thereby calculate the quantity of iron bound.

METHODS

A. *Solutions and Reagents.*—

1. Carrier-Radioiron Solution: This was prepared from $FeCl_3$ solution (as Fe wire dissolved in HCl) and from appropriately diluted radioiron, as $Fe^{59}Cl_3$.* The solution was so prepared that, when eventually reduced (Solution 2, below), it contained, in 0.05 ml., approximately 6.0 μg of ferrous iron and approximately 0.02 microcurie of radioactivity.

2. Reduced Carrier-Radioiron Solution: This was prepared fresh daily by adding to 1.0 ml. of solution No. 1, 60 mg. of pure, crystalline ascorbic acid, freshly opened from a sealed bottle. This quantity of ascorbic acid was approximately 300 times the amount theoretically needed to reduce all the ferric iron present.

3. Neutralized 100 per cent saturated ammonium sulfate: Concentrated NaOH was added dropwise to a saturated solution of ammonium sulfate (53 Gm. diluted to a solution volume of 100 ml. at room temperature) until a pH between 7.0 and 7.5 was obtained, as determined by a Beckman pH meter or by an appropriate chemical indicator.

B. *Procedure.*—

All glassware, with the exception of routine hospital needles and syringes, was rendered iron free by overnight immersion in cleaning solution, followed by multiple washings successively in tap, distilled, and glass-distilled water. Venous blood was obtained, without hemolysis, in a syringe coated with mineral oil. The clotted blood was centrifuged at 1,200 G. for twenty minutes, and the serum was decanted and centrifuged again to eliminate all remaining erythrocytes.

One milliliter of serum was placed in a 50 ml. Erlenmeyer flask and to it was added 0.05 ml. of the reduced carrier-radioiron solution. The mixture was well shaken, the flask was tilted to pool the fluid, and it was allowed to stand approximately fifteen minutes at room temperature. Iron-free materials were not necessary beyond this point.

*Supplied by the Oak Ridge National Laboratory and prepared by neutron bombardment of electromagnetically enriched Fe^{58}.

Approximately 20 ml. of the neutralized saturated ammonium sulfate solution was then added, using a widemouthed pipette to allow almost instantaneous delivery. The flask was stoppered and allowed to remain at least five hours, or preferably overnight.

The suspension was then filtered into a 90 ml. centrifuge tube using fluted Whatman No. 50 filter paper, and the first few drops of filtrate were returned to the funnel for refiltration. The emptied Erlenmeyer flask was washed four times with 5.0 ml. of the ammonium sulfate solution and the total fluid volume was then poured into the filter paper to wash the precipitate. The filter paper with its precipitate was discarded after it had drained completely, and the remaining apparatus was rinsed with distilled water, the washings being added to the filtrate. For counting, the filtrate in the 90 ml. tube was diluted to approximately 50 ml. with ordinary distilled water and then counted in a "Texas well" counter. For scintillation counting, an appropriate aliquot may be counted without drying in a well-type, thallium-activated, sodium iodide crystal. Appropriate standards are prepared in similar fashion for each counting technique.

The determination of the total iron in the standard solution was done by diluting 0.05 ml. of the reduced carrier-radioiron solution to 2.0 ml. with iron-free water, and then measuring iron content according to the method of Barkan and Walker.[9] The protein-bound iron, in gamma per cent, was calculated by multiplying the difference between the radioiron added and the radioiron remaining in the filtrate by the specific activity of the standard solution. The formula is thus:

$$\text{Iron-binding Capacity} = (\text{Radioiron added} - \text{radioiron in filtrate}) \times \frac{\left(\frac{\text{Total iron}}{\text{in standard}}\right)}{\left(\frac{\text{Radioiron}}{\text{in standard}}\right)} \times 100 \text{ (gamma \%)}$$

DISCUSSION

As the agent for separation of protein-bound iron from unbound iron neutralized saturated ammonium sulfate was selected following theoretical consideration, actual empirical use, and later rejection of numerous other techniques. Dialysis, ion-exchange procedures, and more elaborate devices were felt to be too complex for routine laboratory use. Acidification methods (e.g., trichloroacetic acid) of protein precipitation lower the pH so abruptly that iron splits from its protein attachment before precipitation; heavy metals (e.g., mercury) act at neutral or basic pH but were not used because of possibility of exchange with the protein-combined iron; denaturation agents (e.g., acetone) cause an increase in the free carboxylic acid groups[10] of protein (to which iron is attached[11]) and, moreover, denaturated protein tends to absorb some of the excess unbound iron. Finally, sodium sulfate[12-14] requires a working temperature of 37° C.

Ammonium sulfate, the agent of choice, is readily soluble at room temperature and it does not alter the iron-protein complex. Since acidic pH has been shown to alter iron-binding properties of serum,[2, 15, 16] the saturated ammonium sulfate is brought to chemical neutrality by the addition of concentrated sodium hydroxide prior to its use. Pilot experiments demonstrated that this may not have been necessary, yet the results were more consistent and theoretical objections were better satisfied when it was done.

There are trace quantities of iron present in laboratory ammonium sulfate which, in the saturated solution, become of sufficient magnitude to prevent the use of colorimetric, rather than radiochemical, techniques. Ideally, were it not for this trace iron in the precipitating agent, the desired procedure could be

performed merely by adding an excess of stable rather than radioactive iron to the protein, then precipitating the protein, and measuring the excess unbound iron colorimetrically. It becomes a necessity, however, rather than a mere technical advantage, that radioiron be used. As will be shown, the protein-bound radioiron is not displaced by the stable iron introduced with the ammonium sulfate.

Since the iron-binding protein is a β globulin, the bound iron may be removed with the globulins by 50 per cent saturated ammonium sulfate,[17] or all the proteins may be removed by greater than 90 per cent saturated ammonium sulfate. Total protein removal was preferred because it avoided the presence, in the filtrate, of any abnormal protein to which iron might be bound in minute amounts. The addition of at least 19 ml. of saturated ammonium sulfate solution to 1 ml. of serum resulted in a solution of over 95 per cent saturation. In an effort to minimize the volumes of solutions used, the 1.0 ml. serum samples were not buffered prior to addition of radioiron standard and ammonium sulfate. Preliminary experiments carried out with and without buffer demonstrated comparable results.

The radioiron used is brought to ferrous form prior to use, because of the known lesser solubility of ferric salts.

One of the critical points in the validity of this technique is to demonstrate that the radioiron added in vitro combined directly with the "unsaturated" bonds of the protein and did not exchange with or displace any of the stable

TABLE I. VARIATIONS IN RADIOIRON UPTAKE BY 1 ML. SERUM FOLLOWING PRELIMINARY ADDITIONS OF VARYING AMOUNTS OF STABLE IRON

	STABLE IRON ADDED (GAMMA)	RADIOIRON ADDED (GAMMA)	RADIOIRON COMBINED TO PROTEIN (GAMMA)	TOTAL IRON COMBINED TO PROTEIN (GAMMA)
Sample 1	0.0	3.20	1.77	1.77
	0.45	3.20	1.21	1.66
	0.90	3.20	0.92	1.82
	1.35	3.20	0.43	1.78
Sample 2	0.0	5.70	2.80	2.80
	0.40	5.70	2.30	2.70
	0.80	5.70	2.05	2.85
	1.60	5.70	1.21	2.81

TABLE II. CONSISTENCY OF IRON-BINDING CAPACITY OF 1 ML. SERUM WHEN RADIOIRON IS EMPLOYED IN VARYING AMOUNTS AND WHEN ADDITIONAL STABLE IRON IS ADDED LATER

	RADIOIRON ADDED (GAMMA)	STABLE IRON ADDED (GAMMA)	RADIOIRON COMBINED TO PROTEIN (GAMMA)
Sample 3	2.56	1.12	1.96
	3.20	1.12	2.09
	3.84	1.12	2.04
	3.20	2.24	2.10
Sample 4	5.70	0.40	2.59
	5.70	0.80	2.68
	5.70	1.60	2.66

iron which was natively attached. Numerous experiments demonstrated that this latter phenomenon did not occur. Among these experiments and their results were the following:

A. Incubation of a fixed amount of radioiron with a fixed amount of serum for increasing periods of time, from 1 minute to 120 minutes, did not significantly alter the constancy of the amounts of radioiron in the protein-free supernatant.

B. A fixed amount of serum was incubated for a fixed period of time with increasing amounts of radioiron, all greater than saturation levels. Results showed essentially identical uptake of radioiron by all the sera samples, regardless of the amount of radioiron added.

C. Different amounts of stable iron, as ferrous ammonium sulfate, insufficient to saturate the protein, were added to the serum, allowed to incubate, and then a fixed, saturation dose of radioiron was added, followed by precipitation of the protein. The order of addition was then reversed, i.e., first the doses of radioiron were added, and then the different amounts of stable iron were added, prior to precipitation. Results are shown in Tables I and II. In Table I, it is shown that the sum of the stable iron added and of the combined radioiron gives essentially the same values for iron-binding capacity in each sample. This could not be so if any of the radioiron displaced the "native" serum iron. Table II shows that the radioiron, once it is added to the serum, remains tightly combined, and is not itself displaced by further addition of stable iron.

From these results we may infer not only that the radioiron does not displace attached "native" iron, but also that the trace qauntities of iron present in ammonium sulfate do not affect the final determination.

RESULTS

Many samples of sera were tested for iron-binding capacity simultaneously by the radioiron method just described and by a slight modification of the technique described by Rath and Finch.[7] The latter method was identical with what they have described, except that an Evelyn Colorimeter with a filter of 515 mμ was used, the 2 ml. samples of serum being diluted to 6 ml., with corresponding 6 ml. samples of saline as controls.

A sampling of the values obtained is listed in Table III. The column labeled "Estimated Reliability," etc., is used to designate how sharply demarcated the graphical point of saturation could be determined from the spectrophotometric data. In cases labeled "Fair" or "Poor," there was no distinct end point, and the value taken from the graphs was the one which seemed to represent most accurately the graphic saturation point, and it was chosen independently of the concomitantly determined radioiron value.

The results demonstrate excellent qualitative agreement between the spectrophotometric and the radioiron methods, in that the low and high value ranges coincided. The exact quantitative agreement is more difficult to assess, par-

TABLE III. SERUM IRON-BINDING CAPACITY IN VARIOUS CLINICAL CONDITIONS: A COMPARISON OF VALUES OBTAINED SIMULTANEOUSLY BY THE SPECTROPHOTOMETRIC (RATH AND FINCH) METHOD AND BY THE RADIOIRON METHOD (SERUM IRON VALUES LISTED WHERE AVAILABLE)

NUMBER	DIAGNOSIS	ESTIMATED RELIABILITY OF SPECTROPHOTOMETRIC METHOD	SERUM IRON-BINDING CAPACITY		SERUM IRON* (GAMMA %)
			SPECTROPHOTOMETRIC VALUE (GAMMA %)	RADIOIRON VALUE (GAMMA %)	
1	Normal male	Good	241	212	—
2	Normal male	Fair	127	213	125
3	Normal female	Fair	127	159	—
4	Essential hypertension	Fair	171	178	44
5	Giant follicular lymphoma	Fair	63	87	200
6	Serum from #5 kept at 5° for 2 days	—	Could not be determined	89	—
7	Duodenal ulcer	Excellent	292	257	25
8	Duodenal ulcer	Good	273	229	34
9	Pernicious anemia (treated)	Good	196	169	50
10	Pneumococcal pneumonia	Good	279	244	—
11	Radiation exposure	Poor	83	130	105
12	Histoplasmosis	Poor	171	227	70
13	Hodgkin's disease	Excellent	190	208	58
14	Diabetes mellitus	Good	190	213	—
15	Ankylosing spondylitis	Poor	127	156	—
16	Acquired hemolytic anemia with jaundice	—	Could not be determined	90	305
17	Ulcerative colitis	Good	495	308	9
18	Chronic lymphatic leukemia	Good	272	278	80
19	Chronic glomerulonephritis (in uremia)	Poor	50	95	212
20	Multiple myeloma	—	Could not be determined	124	330

*Determined on the original serum by the method of Barkan and Walker.

ticularly since, as noted, the spectrophotometric readings were occasionally of uncertain reliability, even when no interfering phenomena existed. The data indicate that:

1. Results obtained by the radioiron method sufficiently parallel those obtained by the spectrophotometric method to presume comparable accuracy.

2. The radioiron method can be used in two important instances where the spectrophotometric method cannot, viz., with jaundiced serum and with serum which is older than twenty-four hours, obtaining a result in the latter which is essentially identical to the original value.

Using the radioiron technique, it is theoretically possible to calculate the original amount of protein-bound serum iron, as well as serum iron-binding capacity. Premise for such calculation is based upon the observation that iron is totally dissociated from serum protein at a pH of 5.0 or below.[2] Thus, if serum is brought to pH 5.0, and surplus radioiron mixed with it at this stage, following which the pH is returned to normal, the protein will recombine with iron to the point of saturation. However, from the pool of originally bound iron plus added radioiron of known specific activity which was present at pH 5.0, the protein will combine with a mixture of stable and radioiron so that in the supernatant solution will be left a quantity of radioiron different from the excess radioiron remaining when the latter is added a regular serum pH, without the pH manipulation. A simple algebraic relationship can be evolved, using

TABLE III—CONT'D

NUMBER	DIAGNOSIS	ESTIMATED RELIABILITY OF SPECTROPHOTOMETRIC METHOD	SERUM IRON-BINDING CAPACITY		SERUM IRON* (GAMMA %)
			SPECTROPHOTOMETRIC VALUE (GAMMA %)	RADIOIRON VALUE (GAMMA %)	
21	Polycythemia secondary to congenital heart disease	Excellent	495	483	—
22	Lymphosarcoma	Good	200	220	95
23	Hodgkin's disease	Fair	150	167	50
24	Probable "collagen" disease	Good	150	171	70
25	Treated pernicious anemia	Good	180	222	85
26	Asthmatic bronchitis	Good	300	247	105
27	Acquired hemolytic anemia; jaundice	—	Could not be determined	141	155
28	Chronic glomerulonephritis (in uremia); silicosis	Fair	175	178	70
29	Lymphosarcoma	Excellent	225	271	60
30	Thyroid carcinoma with metastases	Poor	94	128	70
31	Obesity	Good	140	255	95
32	Metastatic carcinoma of colon	Good	175	200	—
33	Metastatic carcinoma of breast	—	Could not be determined	83	70
34	Lymphosarcoma	Excellent	170	216	70
35	Treated pernicious anemia; cellulitis	Fair	200	256	100
36	"Banti's syndrome" with eosph. varices	Excellent	225	255	60
37	Lipoid proteinosis	Good	200	203	107
38	Hypochronic anemia due to metrorrhagia	Excellent	200	311	15
39	Postphlebitic chronic leg ulcer	Good	200	204	44

*Determined on the original serum by the method of Barkan and Walker.

the different amounts of radioiron remaining in the supernatant in the two cases, i.e., with and without pH manipulation, to calculate the magnitude of the original, protein-bound iron. Experiments are in progress to implement the practical application of this technique.

SUMMARY

A new method for determining iron-binding capacity of human serum is described and the theoretical and experimental aspects of its validity are discussed.

The method has quantitative and qualitative advantages over the existing spectrophotometric technique, chiefly in the wider range of the types of sera to which it is applicable and the greater accuracy of measurement which it provides.

A proposed application of the radioiron technique for calculating original serum iron is suggested.

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A NOTE ON THE AZOALBUMIN METHOD FOR THE DETERMINATION OF PEPTIC ACTIVITY

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IN THE routine analysis of the gastric contents of patients with peptic ulcers before and after a variety of operative maneuvers, we have been measuring both acidity and peptic activity.[2] Peptic activity is evaluated by the photometric method described by Tomarelli, Charney, and Harding,[5] which utilizes azoalbumin as the substrate. This diazotized protein when precipitated with trichloroacetic acid yields a colorless filtrate, but after proteolytic digestion produces colored reaction products which are soluble in trichloroacetic acid. Since the color intensity relationship of azoalbumin and its digestion products follows Beer's law, a measurement of proteolytic activity is obtained by substituting optical density values for substrate concentrations.

Occasional inconsistencies in our initial results prompted re-examination of the original procedure. Although our findings are essentially in accord with those of Tomarelli and his associates, we wish to stress several technical points which may be overlooked and lead to abnormally low values.

EXPERIMENTAL

A. *The Effect of Increased Peptic Activity on the Kinetics of the Reactions.*—It was found that with samples of high peptic activity, using our azoalbumin substrate, the reaction was no longer first order. This deviation was confirmed by digesting samples of substrate with varying concentrations of gastric juice or granular pepsin, for specified time intervals. Fig. 1 shows the deviation from first order rates when the log ratio of the initial (C_1) and final (C_2) concentrations of undigested substrate exceed approximately 0.04. This effect can also be demonstrated by plotting increasing dilutions of gastric juice against log C_1/C_2 after a thirty-minute incubation period (Fig. 2).

B. *Substrate and pH Effects.*—In the original procedure[5] an initial substrate concentration of 25 mg./ml. is used. Its color intensity and that of the digestion products are measured after intensification by the addition of sodium hydroxide. Deviations from the pH specified for development of the color produced marked changes in color intensities. Thus when varying the pH from 10.3 to 11.5, Klett readings are increased from 101 to 150, while at pH 12.0 ± 0.3 (the suggested pH range) the optical density remained fairly constant at 170.

By decreasing the substrate concentration and keeping the digestion time constant it was found that in the lower concentration levels studied (from 25 to 12.5 mg./ml.) the log ratio $\frac{C_1}{C_2}$ increased slightly. However, at higher concentrations (from 25 to 45 mg./ml.) the log ratio steadily decreased from 0.024 to 0.008.

DISCUSSION

Previous investigators[1, 3, 4] have commented upon the inhibition of pepsin reactions, caused presumably by the accumulation of digestion products, and

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This investigation was supported in part by a research grant (G-3394 C) from the National Institute of Health, Public Health Service.

Received for publication, Aug. 13, 1953.

have stressed the importance of dilution in minimizing this effect. Northrop[4] has stated that the peptones formed during the reaction combine with the enzyme to produce an inactive pepsin-peptone complex. Thus, in samples with high peptic activity, the decreased reaction rate will produce erroneously low values.

While our studies have shown this same effect when log C_1/C_2 exceeds 0.04, Tomarelli and associates[5] have reported a linear relationship to approximately 0.06. This discrepancy may have been caused by the inherent differences be-

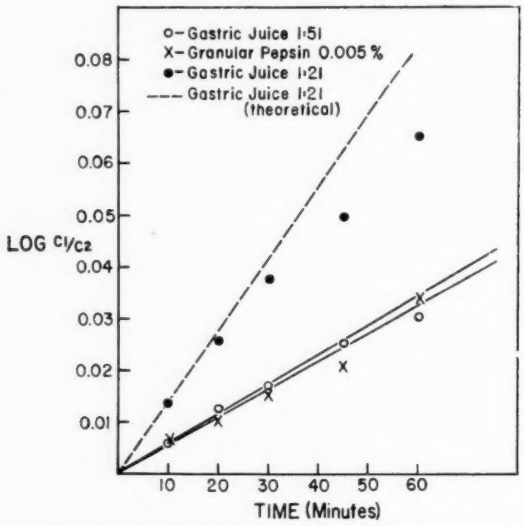

Fig. 1.—Relationship of log C_1/C_2 to time of digestion.

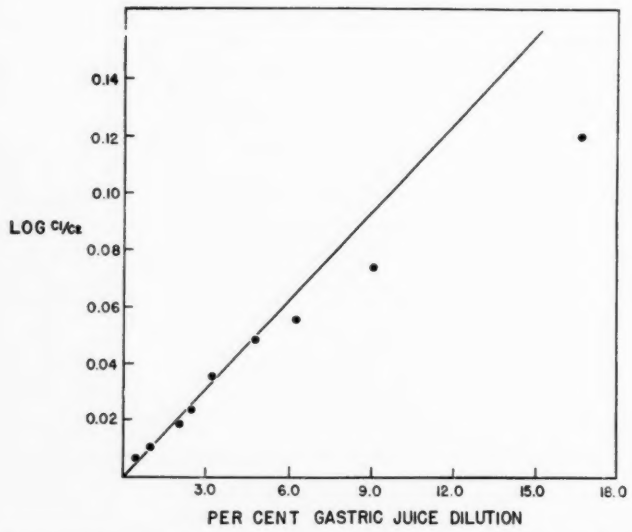

Fig. 2.—Relationship of log C_1/C_2 to percentage of gastric juice dilution (30-minute incubation).

tween preparations of the same substrate. However, our results have been consistent, using three lots of the substrate prepared at different times during the past two years.

To overcome the error resulting from deviations from first order rates, we have modified the original method by routinely diluting all gastric samples 1:21 and incubating for ten minutes. If the log ratio exceeds 0.01, each sample is appropriately diluted so that after incubation for thirty minutes the log ratio falls below 0.04.

The decrease in log ratio obtained by increasing substrate concentration above the indicated level is a typical example of substrate inhibition. Thus to minimize possible errors from substrate and pH effects, the conditions as specified in the original procedure should be adhered to as closely as possible.

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PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Twenty-Sixth Annual Meeting

Chicago, Ill., Oct. 30 and 31, 1953

ABSTRACTS—Concluded

86. TREATMENT OF THE NEPHROTIC SYNDROME WITH NITROGEN MUSTARD AND ACTH

FREDERIC A. LESTINA, M.D. (BY INVITATION), SMITH FREEMAN, M.D., PH.D., AND HENRY WILSON, M.D. (BY INVITATION), CHICAGO, ILL.

This report is concerned with the effects of nitrogen mustard and ACTH therapy on seven patients with nephrosis and extensive proteinuria for three to twenty-four months prior to study. There were four male and three female patients, with ages ranging from 4 to 37 years. The patients were studied in the Metabolic Unit of Wesley Memorial Hospital. The diets were kept constant as to sodium, protein, and calorie content. Laboratory observations consisted of blood electrolytes, cholesterol, electrophoretic patterns, bone marrow aspirations and hemograms, urinary protein and ketosteroid excretion, and endogenous creatinine clearance. Five patients received ACTH before, during, and after mustard therapy. A single course of mustard therapy consisted of 0.1 mg. per kilogram of body weight intravenously daily for four consecutive days. All of the patients received at least two such courses, and two patients have received four courses of nitrogen mustard. Five of the seven patients showed an excellent response to treatment in that during the course of therapy, or shortly thereafter, their urine became protein-free (four patients) or contained less than 0.1 Gm. of albumin per 24-hour specimen. Three of these patients have remained protein-free for one year and two recent patients have been protein-free for two months. After four series of therapy one patient showed an 80 per cent reduction in albuminuria and one patient, on the basis of albuminuria, has showed no response after four series of mustard therapy. In all patients that responded to treatment the initial hypercholesterolemia returned to normal and serial electrophoretic patterns showed a decrease in the alpha 2 and beta fractions associated with a rise in the serum albumin.

87. A FAMILY WITH HOMOZYGOUS HEMOGLOBIN C AND SICKLE CELL TRAIT UNION: A CLINICAL, HEMATOLOGICAL AND ELECTROPHORETIC STUDY

WILLIAM C. LEVIN, M.D., ROSE G. SCHNEIDER, PH.D. (BY INVITATION), JOHN A. CUDD, B.S. (BY INVITATION), AND JOHN E. JOHNSON, JR., M.D. (BY INVITATION), GALVESTON, TEXAS

The case of a Negro woman homozygous for hemoglobin C has been characterized clinically and hematologically. The husband has sickle-cell trait. Of three children, one has sickle-cell hemoglobin C disease (S and C hemo-

globin) and the others have hemoglobin C trait (normal and C hemoglobin).

The index case (R. H.), the 17-year-old son, reported frequent episodes of abdominal cramping, joint pains, and chronic jaundice. Physical examination revealed icteric sclerae but no hepatosplenomegaly. A normal erythrocyte level, slight hypochromia, slight reticulocytosis, minimal sickling, extremely numerous target cells, and pronounced erythroid stimulation in the marrow were observed. Electrophoretic analysis of the hemoglobin confirmed the clinical impression of the presence of S and C types.

The two younger siblings were asymptomatic. A moderate number of target cells but absence of sickling were observed in the blood of each. Electrophoretic studies of the hemoglobin of each revealed a mixture of C and normal hemoglobin.

The father, a 41-year-old Negro, was asymptomatic. Minimal sickling was observed. No target cells were seen. Electrophoretic studies of his hemoglobin showed a mixture of normal and sickle-cell types.

The mother, a 38-year-old Negro, was diagnosed as having anemia following the first of three normal pregnancies. Cholelithiasis was recognized in 1950. No history of icterus was elicited. Presently, the only complaints were mild weakness and fatiguability. Her family history was noncontributory. Physical examination revealed splenomegaly (spleen 10 cm. below the rib margin). The leukocyte count was normal with a granulocytic left shift. A moderately severe normochromic anemia (R.B.C. 3,070,000; hemoglobin 9.6 Gm. per cent) was present. Moderate numbers of target cells were seen, but no sickling was demonstrated. Reticulocytes were normal. The marrow showed a tremendous erythroid stimulation of the type found in hemolytic anemia.

Hemoglobin samples of the members of this family were analyzed by paper electrophoresis, simultaneously with normal and sickle-cell anemia samples as controls. The resulting data illustrate genetic relationships between normal, S and C types of hemoglobin, and exemplify almost all the known combinations of these types. Analysis of hemoglobin samples of these patients in the Tiselius electrophoresis apparatus showed interesting quantitative relationships in the inheritance of the three types of hemoglobin.

88. THERAPEUTIC EVALUATION OF CORTICOTROPIN (ACTH) AND CORTISONE IN FORTY CASES OF IDIOPATHIC THROMBOCYTOPENIC PURPURA

Louis R. Limarzi, M.D., and William R. Best, M.D.
Chicago, Ill.

Corticotropin was given to 12, cortisone to 14, and both agents to 14 cases of idiopathic thrombocytopenic purpura. Doses were within the usual therapeutic range for age and route of administration. All platelet counts prior to therapy were less than 100,000 per cubic millimeter. Ages were from 3 months to 69 years. Twenty-six were females. Twenty-five cases were acute; 15 were chronic. Four patients had previous splenectomy with no effect or later relapse. One of these had a lasting remission on cortisone, one a temporary remission, and two no effect from either drug. Of the remaining 36 cases, 17 had no effect from these agents, 11 had a temporary remission, 7 had a lasting remission, and one had a lasting remission to a second course of cortisone despite failure of response to the first. Of 12 given only corticotropin, 5 had a lasting and 6 a temporary remission. Of 11 given only cortisone, 2 had a lasting and 4 a temporary remission. In only 1 of 12 un-

responsive cases so tested did the two agents act differently—corticotropin but not cortisone induced temporary remission. Eight of 12 chronic and 11 of 26 acute cases had remissions with these agents; however, remissions were sustained in only 2 chronic cases and 6 acute cases. Three of 4 deaths were in the acute cases. There appeared to be no relation between the number and maturity of megakaryocytes and the response to these agents. Thirteen patients with no response were splenectomized; 7 showed a lasting remission; 6 had no effect from this procedure or subsequent corticotropin and cortisone treatment. Nine patients showing temporary remission to these agents were splenectomized; 8 had lasting remissions; one showed no response to either drug. Postsplenectomy platelet counts were often quite high when patients received these agents preoperatively. The bleeding tendency was occasionally improved without increase in platelets. In summary, these agents are of some value in about half the cases of this disease; corticotropin appearing slightly better than cortisone. They are especially useful in preparation for splenectomy.

89. THE CEREBRAL AND RENAL HEMODYNAMIC RESPONSES TO BLOOD PRESSURE REDUCTION TO HYPOTENSIVE LEVELS BY GANGLIONIC BLOCKADE WITH HEXAMETHONIUM, ARFONAD, AND PENDIOMIDE AS USED FOR HYPOTENSIVE SURGERY IN NORMOTENSIVE HUMAN SUBJECTS

W. R. Livesay, M.D. (By Invitation), John H. Moyer, M.D., George Morris, M.D. (By Invitation), and Harvey Snyder, M.D. (By Invitation), Houston, Texas

Because of the increasing use of ganglionic blocking agents for reducing the blood pressure to hypotensive levels for certain surgical techniques the effect of this procedure on the renal and cerebral circulation was studied in normotensive human subjects. Cerebral blood flow was measured using the nitrous oxide technique. Renal plasma flow was estimated by the para-aminohippurate clearance technique and glomerular filtration was determined by inulin clearance. After suitable control periods Pendiomide, hexamethonium, or the thiophanium derivative, Arfonad, was administered by continuous intravenous infusion until maximum reduction in blood pressure was obtained. After hemodynamic stabilization, observations were again made on cerebral and renal hemodynamics. The results indicated that Arfonad was the most potent hypotensive agent. The renal response showed an initial and prolonged reduction in glomerular filtration rate by all three agents but was most marked in the case of Arfonad. Whereas renal blood flow was initially depressed by the three agents, this function returned to control levels after one hour of maintained hypotension with hexamethonium and Pendiomide. In contrast, the renal blood flow continued to be depressed during hypotension due to Arfonad. This was probably a result of greater blood pressure reduction with Arfonad and consequently a depression to levels at which adequate vasodilation was not possible. The cerebral hemodynamic effect of these agents was to reduce cerebral blood flow and decrease oxygen uptake. This response was most marked when Arfonad was employed, again this was probably due to the more potent hypotensive activity, and due to the fact that vasodilation did not occur to the degree which would be necessary to compensate for the more drastic reduction in blood pressure as compared to that which was observed after hexamethonium and Pendiomide.

90. SERIAL BLOOD AND BONE MARROW STUDIES IN TWO CASES OF METHIMAZOLE (TAPAZOLE) GRANULOCYTOPENIA

JOHN LOUIS, M.D. (BY INVITATION), WILLIAM R. BEST, M.D., AND LOUIS R. LIMARZI, M.D., CHICAGO, ILL.

Seven cases of granulocytopenia with two fatalities due to methimazole have previously been reported. During three years of extensive clinical use we have observed two severe hematologic reactions following use of this drug.

A 52-year-old white woman received 300 mg. of propylthiouracil daily for seventy-eight days, no medication for two weeks, and 30 mg. of methimazole daily for eighty-five days. She developed fever, sore throat, and malaise. At the beginning of methimazole therapy the white blood count was 4,600 with 43 per cent neutrophils. On admission, one week after she stopped medication, the white count was 1,000 with 20 per cent neutrophils. The bone marrow was hypercellular, with a maturation arrest at the progranulocytic stage. On antibiotics and general management the patient had a clinical recovery in a few days, and the white blood count was normal by the end of the first week. The bone marrow was normal eleven days after admission.

A 24-year-old Chinese woman received 30 mg. of methimazole daily for fourteen days, following which she developed a brief febrile episode with skin rash. Following two days with no therapy she was given 15 mg. of methimazole daily for seven days. Another erythematous eruption appeared and persisted for six days following which she was given two doses of 500 mg. of propylthiouracil. A slight tendency for leukopenia was present from the onset of the first rash. The white blood count went down to 3,300 during one phase of methimazole therapy, and to 3,200 with 7 per cent neutrophils following propylthiouracil. At the time of the later count the bone marrow was hypercellular with an increased myeloid-erythroid ratio and a marked shift to the left in the granulocytic elements. One month following thyroid surgery the bone marrow and peripheral blood counts were normal.

On the basis of these two cases it appeared that evaluation of toxicity rested equally on study of the blood, bone marrow, and clinical findings. These toxic manifestations might have been discovered sooner had the patients been properly briefed on potential toxic symptoms.

91. TREATMENT OF POLYCYTHEMIA VERA WITH TRIETHYLENE MELAMINE (TEM)

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Twenty-three cases of polycythemia vera were treated with triethylene melamine (TEM). Thirteen patients were males; one female was colored. Eighteen of these cases were treated with TEM alone for periods of one to thirty-six weeks. Seven had received prior venesection. Initial hematocrits varied from 41 to 61 per cent. TEM was given in doses of 5 to 10 mg. per week for a total dosage of 4 to 360 mg. Most patients received 20 to 260 mg. over a two to twelve-week period. During the period of therapy one case had a 6 per cent increase in hematocrit, 5 cases had no significant changes, 3 cases had 2 to 4 per cent decrease, 8 cases had 5 to 10 per cent decrease, and one case had a 22 per cent decrease. The latter occurred during eight weeks of therapy for a total dose of 100 mg., and was sustained without treatment

for eight months. There was no apparent relationship between initial hematocrit or total dose and degree of response. Five cases have maintained remissions for two to eight months without treatment. The remainder have shown no significant increase of hematocrit during sustained therapy.

Five patients received TEM associated with venesection. They have responded well to this combined therapy.

Two patients of these 23 had abdominal discomfort with treatment. Otherwise in this series and in 29 other hematologic disorders treated with comparable doses of TEM we have noted no untoward effects. These disorders included Hodgkin's disease, lymphosarcoma, chronic granulocytic leukemia and chronic lymphatic leukemia.

When this series is compared with 44 cases of polycythemia vera treated with radioactive phosphorus the latter appears to be a more effective method of therapy.

92. EFFECT OF SPECIFIC ANTIBODY ON THE PHAGOCYTOSIS OF VIRUS MODIFIED R.B.C.

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Normal human, rabbit, and chicken erythrocytes were not phagocytized to any significant degree when tested by a recently standardized erythrophagocytic technique using tissue culture macrophages (J. Lab. & Clin. Med. **41**: 169, 1953). Many types of in vitro modifications have the capacity to "opsonize" normal red blood cells so that they are readily phagocytized. Among these "opsonizing" modifiers are: trypsin and other proteolytic enzymes, specific and isohemagglutinins, and various viruses. Red blood cells of some patients with malignancies, certain infectious diseases, and hemolytic anemias are also more readily phagocytized than normal R.B.C. The following studies were part of a continuing inquiry into the fundamental factors influencing the susceptibility of R.B.C. to phagocytosis.

(1) Red blood cells from chickens experimentally infected with Newcastle disease virus (N.D.V.) were phagocytized to the same degree as chicken R.B.C. treated with N.D.V. in vitro.

(2) Normal human group O and normal chicken erythrocytes were modified in vitro by treatment with active N.D.V. for several hours at 37° C. These modified R.B.C. were used to immunize rabbits for the production of an hemagglutinin with specificity for the virus-treated R.B.C. By absorption techniques, serum fractions were obtained and shown to specifically agglutinate erythrocytes treated with N.D.V. in vitro, as well as R.B.C. from N.D.V. infected chickens. Thus, there appears to be a relation between the erythrocyte modification detected by the antiserum and that detected by the phagocytic test.

(3) Phagocytic indices (P.I.) for untreated normal group O human R.B.C. and normal untreated chicken R.B.C. were about 10 per cent. The P.I. of these erythrocytes after treatment with N.D.V. were increased to a range of 65 to 75 per cent. After further treatment with absorbed anti-virus-treated-erythrocyte serum, the N.D.V. modified R.B.C. gave P.I. of about 10 per cent. Normal rabbit serum had no effect on the P.I. (70 per cent) of N.D.V. treated red blood cells. This specific hemagglutinin, then, inhibited the phagocytosis of N.D.V. "opsonized" chicken erythrocytes.

In contrast to the usual "opsonizing" effect of antibody, this immune hemagglutinin reversed the enhanced susceptibility to phagocytosis resulting from the modification of R.B.C. by virus.

93. METABOLIC STUDIES OF LAENNEC'S CIRRHOSIS: THE EFFECT OF ORAL ADMINISTRATION OF POTASSIUM

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Although the metabolism of sodium in Laennec's cirrhosis has been investigated intensively, relatively little attention has been directed toward potassium metabolism in this disease. As part of a study concerned with potassium balance in cirrhosis, potassium citrate was administered orally in large doses—390 to 578 meq. of potassium per day—to six cirrhotics, four of whom had ascites and edema. Measurements of sodium, potassium, and nitrogen intake and excretion were made in three patients, and partial balance studies were done in the remaining three. In each study, therapy with potassium citrate was given for seven days. This was preceded and followed by pre- and post-therapy observations, each of which lasted one week. The medication was well tolerated in all patients, and potassium intoxication was not observed, neither clinically nor by laboratory findings. With therapy, small changes in the serum and ascitic fluid electrolyte levels (Na, K, Cl, P, Mg, and Ca) were noted. A sharp and prompt increase in the urinary excretions of sodium, potassium, and chloride and a fall in urinary phosphorus occurred during the therapy period. The biochemical data returned to pre-therapy levels when the medication was stopped. In all patients, the potassium balance became positive during the period of therapy. The nitrogen balance was unchanged during the study. With the exception of changes in the urinary excretion of electrolytes, with their accompanying diuresis, there were no discernible alterations in the course of the disease during the brief study period. In two of the decompensated subjects there was continued diuresis after therapy was stopped. The results of the investigation shall be presented and the findings discussed.

94. EARLY ATYPICAL MANIFESTATIONS OF LEUKEMIA

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The early manifestations of leukemia may be atypical and precede the onset of clinically recognizable leukemia by several years. The purpose of this report is to present the data on eleven patients with similar but atypical hematologic manifestations, eight of whom developed leukemia. These cases were followed from one to five years. In addition to those developing leukemia, one developed reticulum cell sarcoma, one died of aplastic anemia, and one died of hemorrhage.

All of these patients had severe anemia, which was usually hemolytic although the reticulocytes were not uniformly increased. Nine cases had leukopenia; in every instance neutropenia was present. Thrombocytopenia was present in ten patients. A hyperactive marrow was uniformly present.

Nine cases underwent splenectomy without significant benefit. Pathologically, the spleen revealed only passive hyperemia without specific morphological lesions; in one patient who subsequently developed leukemia, myeloid metaplasia was described in the spleen. Following splenectomy there was an increase in circulating normoblasts in all cases and in six patients a significant increase in leukocytes occurred. Therapy with cortisone or ACTH was ineffective.

Of the eight patients who developed leukemia, six were myeloid and two were monocytic in type. In each instance the clinical course was rapid after the development of clinically recognizable leukemia. Many features of these cases resemble "hypersplenism" or a syndrome previously described as "aplastic anemia with a hyperplastic marrow." However, these cases differ from the hypersplenic form of panhematopenia in that the spleen is not usually enlarged. In addition the anemia and thrombocytopenia are more severe than usually encountered in this disease and there is no response to splenectomy. There has been considerable doubt that "aplastic anemia with hyperplastic marrow" represents a distinct entity.

This syndrome of severe anemia, a hyperactive marrow, neutropenia usually with leukopenia, thrombocytopenia, and a normal or only slightly enlarged spleen does not respond to splenectomy. In these cases pathologic examination except in one instance revealed no lesion suggestive of leukemia. It is important to recognize that these unusual hematologic manifestations frequently are a precursor to leukemia and are not a form of hypersplenism or aplastic anemia.

95. THE EFFECT OF EATING UPON THE ABSORPTION OF VITAMIN A

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The absorption of vitamin A in oily solution, given orally to subjects in the fasting state, has been accepted generally as a measure of fat absorption. When a test dose of 100,000 micrograms of vitamin A palmitate was administered in this manner to 16 apparently healthy subjects, the serum vitamin A levels of 5 subjects failed to rise appreciably during a six-hour period. In these same 5 subjects, ingestion of food two hours after administration of the test dose produced a rapid rise in the serum concentration of vitamin A.

When this same test dose was administered to 48 fasting hospitalized subjects, ingestion of food two hours later produced in all a marked acceleration of the rate at which the serum vitamin A concentration had been rising. These findings prompted a study of the possible roles of the stomach and small intestine in vitamin A absorption, as follows:

(1) Three totally gastrectomized subjects showed the same response to eating as did the normal subjects.

(2) Three normal subjects demonstrated the same response to sham feeding as to actual ingestion of food.

(3) Six normal fasting subjects received vitamin A intraduodenally, with little evidence of absorption in the following 4 hours. When the test was repeated, ingestion of food was followed by the expected rise in serum vitamin A levels. This effect of eating could not be blocked by parenterally administered atropine.

(4) In two subjects 12 inches of upper jejunum were "isolated" by inflated recording balloons, and vitamin A was instilled into the iso-

lated loop. No absorption could be detected until after the subjects took food by mouth.

(5) Thirteen patients with steatorrhea due to various causes failed to absorb the test dose even after eating.

The significance of these results will be discussed, and a new procedure for the vitamin A tolerance test will be described.

96. THE STUDY OF THE EARLY DISAPPEARANCE OF IODINATED (I^{131}) SERUM ALBUMIN FROM THE CIRCULATION OF EDEMATOUS SUBJECTS BY A CONTINUOUSLY RECORDING METHOD

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Cleveland, Ohio

The early disappearance of iodinated (I^{131}) serum albumin from the circulation of 10 subjects with generalized edema was studied by a previously described technique utilizing the flow of blood through an external A-V fistula, past a continuously recording scintillation counter head. The disappearance curve of the iodinated (I^{131}) serum albumin was recorded continuously for one to two hours after injection and replotted semilogarithmically for calculation of disappearance rate. Cardiac outputs were done at the beginning of each study by the dilution method of Hamilton, modified for radioisotopes. The urine was assayed for radioactivity at the end of each study.

Of the 10 patients studied, 6 had edema of cardiac origin, 3 of renal origin, and 1 due to Laennec's cirrhosis. All subjects had severe, visible edema and some patients also had ascites and pleural effusions. Contrary to previous speculation by some investigators, the loss of the tagged albumin from the circulation of these edematous subjects during the first twenty minutes after injection was no greater than that found in our previous study of normal subjects by the same method. The average percentile loss at ten and twenty minutes was 1.15 per cent and 2.23 per cent, respectively, regardless of the etiology of the edema.

In the cardiac patients the percentile loss during the entire study period was no greater than that found previously in normals. In the 3 subjects, however, with renal edema and massive albuminuria, the average percentile loss was somewhat greater at the end of the study and urinary assay showed radioactivity in excess of our average normal.

The mean time of "complete" mixing of the tracer substance was prolonged, averaging 14.3 minutes except in the patient with cirrhosis, whose mixing time was five minutes. Although the cardiac output and index varied, the average value of the group was low. The total blood volume, calculated by extrapolation of the semilogarithmic plot over the entire study period, was uniformly elevated in all cardiac subjects, and low or low normal in those patients with noncardiac edema.

97. NASAL INSTILLATION AND INHALATION OF CRYSTALLINE B_{12} IN THE TREATMENT OF PERNICIOUS ANEMIA

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Twelve cases of pernicious anemia in relapse were treated with vitamin B_{12} by nasal instillation or inhalation. Three of these patients received

crystalline B_{12} in physiologic saline by vaponephrin nebulizer. B_{12} lactose powder was administered to one patient by a dust inhalator. Nasal instillation of vitamin B_{12} in saline was utilized in the last 8 cases.

Twenty patients with pernicious anemia in remission were given inhalation and nasal instillation therapy. They were treated at comparable intervals to parenteral maintenance therapy.

Inhalation, nasal instillation, and direct administration of B_{12} into the pulmonary tract resulted in urinary excretion of detectable amounts of the vitamin. Mucous membrane irritation or sensitization have not been encountered to date.

Complete clinical and hematologic remission was obtained in all twelve patients treated by this technique. Twenty patients with pernicious anemia in remission have been maintained by inhalation and nasal instillation of B_{12} for eighteen months. This simple method of therapy is an effective, economical, and safe mode of treatment for pernicious anemia.

These data will be illustrated by clinical charts, photomicrographs, and motion picture.

98. A COMPARISON OF RESULTS IN THE TREATMENT OF HYPERTENSION WITH RAUWOLFIA SERPENTINA ALONE AND IN COMBINATION WITH HEXAMETHONIUM OR HYDRALAZINE

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A group of 158 patients with hypertension have been studied on an ambulatory outpatient basis. All patients had an average control blood pressure of not less than 160 systolic and 110 diastolic. Eighty-five of the patients received *Rauwolfia serpentina* (Rauwiloid) alone. Forty-five of the 85 patients were responsive to Rauwolfia alone with a fall in mean blood pressure of 20 mm. Hg or more and 19 of these became normotensive with a fall in mean blood pressure to 110 mm. or less. More severe cases and patients with complications due to hypertension were found to be far less responsive than the milder uncomplicated cases. Side effects were all benign. Hydralazine was added to 11 patients who were unresponsive to *Rauwolfia serpentina* alone after which 8 became responsive. Rauwolfia ameliorated the side effects of hydralazine and controlled the pulse-accelerating action. Four in a group of 9 additional patients who were started on combined Rauwolfia and hydralazine therapy failed to tolerate the latter agent. The remaining 5 responded to treatment. Only 3 patients out of 64 patients treated with combined hexamethonium and *Rauwolfia serpentina* failed to respond and 39 of the responding patients became normotensive in spite of a high incidence of hypertensive complications prior to therapy. Retinopathy was found to be uniformly reversible in 21 cases with this complication. Not only was the hypotensive effectiveness of hexamethonium augmented but the dose requirement of this agent was reduced and side effects became less intense and more tolerable. No serious side reactions occurred. It was concluded that *Rauwolfia serpentina* is an effective hypotensive agent in mild uncomplicated hypertension and that a combination of hexamethonium and Rauwolfia can be expected to control the majority of severe complicated cases of hypertension. The mild and moderately severe cases of hypertension which do not respond to *Rauwolfia serpentina* alone will frequently obtain a significant reduction in blood pressure when hydralazine is added to the therapy.

However, it is desirable to first treat the patient with Rauwolfia in order to block off the untoward reactions which otherwise accompany hydralazine administration.

99. RELATIVE INCIDENCE OF GOUT IN NEGRO AND WHITE MALES AT COOK COUNTY HOSPITAL (1944-1952)

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The literature at the present time reveals few extensive reports on gout in the Negro male. Well-recognized authorities in this field have noted the need for more careful and complete statistical analysis of the relative incidence of gout in the Negro male. It is generally accepted that figures recording the incidence of gout in all races in all parts of the world range between less than 1 per cent and 7 per cent.

It is the purpose of this report to contribute a part of the much needed statistical data for analyzing the relative incidence of this disease in the Negro male. The record library and the men's ward at the Cook County Hospital, Chicago, Ill., provided the material for this investigation.

DATE	ADMISSIONS TO MALE MEDICINE	GOUT IN WHITES	GOUT IN NEGROES
1944	8,180	3	1
1945	8,957	3	0
1946	10,287	6	1
1947	11,386	2	1
1948	11,680	3	4
1949	11,755	10	4
1950	11,167	12	3
1951	10,760	4	5
1952	10,051	6	2

A breakdown of the total male medicine admissions to white and Negro race reveals that 35 to 40 per cent were Negro with a gout incidence ranging from 0.06 to 0.1 per cent. The White race averaged between 60 to 70 per cent of the total admissions with a gout incidence ranging from 0.04 to 0.2 per cent.

100. ON THE PATHOGENESIS OF HEMOLYSIS IN HEMOLYTIC ANEMIA ASSOCIATED WITH COLD HEMAGGLUTINATION

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(INTRODUCED BY WILLIAM W. ENGSTROM, M.D.)

The association of hemolytic anemia with cold hemagglutination is well known, but the mechanism of hemolysis has not been completely elucidated. Stats (Proc. Soc. Exper. Biol. & Med. **54**: 305, 1943) demonstrated increased mechanical fragility of cold agglutinated erythrocytes and concluded that hemolysis was a mechanical rather than an immunologic phenomenon. On the other hand, Ferriman et al. (Quart. J. Med. **79**: 275, 1951) described a cold hemolysin in their three cases.

A man, aged 80, with chronic, moderately severe hemolytic anemia had a cold agglutinin in a titer of 1 to 4,096 and a negative Coombs antiglobulin test. He was treated with cortisone and made a symptomatic recovery. Following self-discontinuance of the drug a severe hemolytic relapse ensued. The cold agglutinin titer rose to 1 to 64,000, while the direct Coombs test became positive. During the performance of the Donath-Landsteiner test, a hemolysin active against iso- cells was noted after thirty minutes of incubation at body temperature. The hemolysin required complement and was partially removed by absorbing the patient's serum with anto- and iso-erythrocytes at 37° C. The hemolytic reaction was enhanced by the use of trypsinized erythrocytes. Titration of the hemolysin was carried out, using heat-inactivated serum by the method of Dacie and DeGruchy in which AB serum was used as diluent and as a source of complement. The titer of hemolysin was found to be 1 to 32 when trypsinized test cells were used.

During the period of excessive hemolysis, the mechanical fragility was 28 per cent (normal fragility: 5-10 per cent). Later treatment with adrenocorticotropic hormone was followed by disappearance of the hemolytic antibody. Concomitantly the mechanical fragility fell to 10 per cent.

It is concluded that the hemolysis associated with the presence of cold agglutinins can be both immunologic and mechanical in origin.

101. SERUM VITAMIN B_{12} CONCENTRATIONS IN PERNICIOUS ANEMIA

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(Introduced by Maurice M. Best, M.D.)

Using the Euglena assay method, observations on the vitamin B_{12} content of serum have been made in ten cases of pernicious anemia in relapse. In all cases activity less than 20 $\mu\mu$g/ml. was found. (Normal range: 86-460 $\mu\mu$g/ml.)

Two cases were given an intramuscular injection of the vitamin. In each there was a rapid rise in both the bound and the free form of the vitamin in the serum.

Five cases were given vitamin B_{12} orally in single dosages from 1,000 to 9,000 μg. In two patients given 1000 μg, one showed no rise in serum B_{12} activity, the other, a slight rise which persisted for a few hours only. Neither case experienced a hematological remission. Three cases were given 3,000, 5,000, and 9,000 μg, respectively. Each case showed a rapid response in serum concentration to the normal range which persisted for several days. These three cases experienced an optimal hematological response. The rise in B_{12} concentration was all in the bound form. No free vitamin B_{12} activity was demonstrated in the serum.

The urinary excretion of vitamin B_{12} for the twenty-four hours following these larger oral doses ranged from 0.134 to 0.45 μg. These figures for urinary excretion are in the range of normal daily excretion.

It is concluded that: (a) The assay of the vitamin B_{12} concentration of serum is a very satisfactory diagnostic procedure in pernicious anemia in relapse. (b) There is a correlation between hematological response following therapy and a satisfactory rise in serum B_{12} concentration. (c) With single oral doses of 3,000 μg or greater, an optimal hematological remission can be obtained in pernicious anemia. In such cases the vitamin is absorbed purely in the bound form. (d) Because following oral therapy there is no increase in the free vitamin in the serum, increased urinary excretion of vitamin B_{12}

does not occur. This is contrary to the findings following intramuscular injection of the vitamin. Apparently, vitamin B_{12} in the free state is excreted in the urine but not vitamin B_{12} bound to serum protein.

102. INTRAVASCULAR HEMOLYSIS, THROMBOCYTOPENIA, AND OTHER HEMATOLOGIC ABNORMALITIES ASSOCIATED WITH ECLAMPTOGENIC TOXEMIA OF PREGNANCY

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The pathogenesis of eclamptogenic toxemia of pregnancy is unknown. Certain hematologic changes have been observed which offer new clues to the understanding of this syndrome. Studies in eleven toxemic women have revealed hemolytic or hemorrhagic phenomena or both.

In three, transient gross hemoglobinemia and hemoglobinuria were present, while in six others a hemolytic process was strongly suspected from the presence of reticulocytosis and a falling hematocrit in the absence of significant blood loss. Five patients showed an increased excretion of urinary urobilinogen and in six the concentration of serum indirect bilirubin was elevated. Nucleated erythrocytes in the peripheral blood were less frequent, being found but three times. Erythrophagocytosis in buffy coat preparations, and slightly increased erythrocyte osmotic fragility were found twice. The direct Coombs test was positive in two cases. These tests became negative within four days. In no case were auto- or isoagglutinins or hemolysins demonstrated, nor was the L.E. test positive.

Transient thrombocytopenia was observed in six patients. The lowest platelet counts obtained in each of these six cases ranged from 27,000 to 80,000/mm.^{3} In the cases with severe thrombocytopenia prolonged bleeding times and abnormal capillary fragility were observed. Following delivery the platelet counts rose above 150,000/mm.^{3} within five days.

Other hemostatic defects were found frequently. The clotting time in silicone tubes was prolonged in eight patients and the clotting time of a mixture of oxalated plasma and thrombin was prolonged in seven of the eleven cases. An abnormally low plasma fibrinogen concentration was found but once.

Platelet counts done at delivery on three infants from two thrombocytopenic women were normal. Erythrocytes from these infants showed no autoagglutination in bovine albumin and were Coombs negative. The presence of an acute hemolytic process and multiple coagulation defects in patients with severe pre-eclampsia and eclampsia and their rapid correction following delivery suggests that these defects are dependent upon the products of conception. The possibility that an immunologic process is involved is suggested but unproved.

103. HEMOPHILIC CONDITION IN THE FEMALE

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Whether hemophilia can occur in the female is still problematic. Recently, the opportunity presented itself to study three girls in whom the characteristic hemophilic defect, deficiency of thromboplastinogen, was established but whose conditions do not appear to be identical and are probably not hemophilic.

E., aged 6 years, has had abnormal bleeding since early infancy. Deep muscular and joint hemorrhages have occurred. Clotting time 35 min., bleeding time 3 min., prothrombin time 12 sec., prothrombin consumption time 8 sec., thromboplastinogen activity time 8½ sec., and platelets 210,000. On mixing her blood with an equal volume of hemophilic blood, no correction occurred, but normal blood corrected her blood quantitatively as much as it did hemophilic blood. Her condition is indistinguishable from true hemophilia both clinically and by laboratory tests. The hereditary history is negative; father and mother and a brother are normal.

L., aged 4 years (previously reported), has a condition similar to that of E., except that her bleeding is milder, her thromboplastinogen deficiency less marked and her prothrombin slightly but persistently lowered and refractory to vitamin K. Father, mother, and a brother are normal and there is no history of hemophilia in the family.

F., aged 6 years, has a severe bleeding condition including frequent epistaxis. She has had many transfusions. Clotting time 8 min., bleeding time 30 min.+, prothrombin time 12 sec., prothrombin consumption time 12½ sec., thromboplastinogen activity time 10 sec., platelets 275,000. Mixing her blood with hemophilic blood causes no mutual correction. Her bleeding is probably due to the combined effect of hypothromboplastinogenemia and the prolonged bleeding time. Father, mother, and one brother are normal, whereas her younger brother (aged 2½ years) has a condition identical to hers.

Like the hemophiliac, these three patients have a hypothromboplastinogenemia which is congenital in origin. The lack of a hereditary history and the presence of other coagulation defects in two of the patients make it doubtful whether such conditions should be classed as hemophilia.

104. INDUCTION AND PATHOLOGIC CHARACTERISTICS OF EXPERIMENTAL STREPTOCOCCUS MITIS ENDOCARDITIS

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Endocarditis caused by *Streptococcus mitis* has not been consistently produced previously in the dog.

Aortico-inferior vena caval anastomoses were completed in 24 dogs by the technique of Lillehei. Antibacterial treatment then was given daily for five days. A seven-day period of stabilization followed.

Daily intravenous injections of broth cultures of *Streptococcus mitis* were then begun. All animals received at least 10 ml. daily of a 24-hour culture in dextrose brain broth until results of blood cultures were positive for ten consecutive days; this required an average of 19 days of injections. Bacterial endocarditis developed in 12 dogs; clinical evidence of valvulitis appeared at an average of 18 days after the beginning of injections. These animals lived for an average of seven weeks after onset of the valvular infection. Necropsy revealed infection of the aorticovena caval fistulas and involvement of one or both left-sided cardiac valves in all 12. In addition, 5 displayed involvement of the right-sided valves. The endocarditis was similar to that in human beings; destructive and reparative phenomena coexisted. *Streptococcus mitis* was recovered from the infected valves and fistulas in all. Emboli occurred in the lungs, brain, kidneys, and spleen. Congestive cardiac failure appeared in 11 of these 12 animals.

Endocarditis did not develop in 12 dogs; results of blood cultures had been variable in 7 of these despite daily injection of *Streptococcus mitis* for an average of 19 days and use of multiple daily doses for two weeks. In these 7 dogs, the fistulas either had closed completely or had become greatly narrowed; evidence of healed infection was noted at the fistulas in all 7 animals at necropsy. Results of blood cultures quickly became persistently positive in the remaining 5 animals in which subacute bacterial endocarditis did not develop. All of them had infected fistulas. Four of them died of pulmonary emboli arising from the infected fistulas on an average of 39 days after initial injections; the other died of subarachnoid hemorrhage six months after injections.

It is concluded that endocarditis caused by *Streptococcus mitis* can be produced in the dog by this method.

105. LOCAL AND LONG DISTANCE TRANSMISSION AND STORAGE OF ELECTROCARDIOGRAPHS AND OTHER LOW FREQUENCY SIGNALS

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By converting the low-frequency signal to a frequency-modulated form, several advantages result: (a) the signal can be transmitted over a wide variety of lines with immunity from many of the common electric disturbances, and (b) magnetic tape can be used for recording and storage. For telephone transmission a center frequency of 1,200 cycles was used with a deviation of plus and minus 150 cycles. This center frequency was selected from an analysis of test and signaling frequencies used in the telephone plant and the characteristics of message telephone facilities. For local or private transmission a center frequency of 5,000 cycles was used. Tests made include a wide variety of telephone circuits up to 3,000 miles distance.

The method is applicable for the transmission of any low-frequency signal and the special application requires only the construction of a suitable transducer. Magnetic tape has many advantages for recording transient physiological observations, since it is compact, can be erased and reused, any desired portion can be transcribed, and the tape can be reviewed with proper cathode ray tube monitors at highly accelerated speeds.

106. EXPERIMENTALLY INDUCED CEREBRAL SYNDROME FOLLOWING THE TEST FEEDING OF ALLERGENIC FOODS

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The clinical manifestations of cerebral allergy are apparently related both to impaired cerebration and to altered motor function. Cerebral reactions in which clinical evidence of depression predominates have been observed repeatedly in performing individual food ingestion tests for the diagnosis of specific food allergy. Although usually occurring in individuals subject to periodic unexplained reactions of this type, they may develop during test feeding in the absence of such a history.

These cerebral responses observed after test feeding most commonly begin with upper respiratory symptoms, coughing, or pruritus, which merge with the subsequent development of fatigue, headache, or muscle aching or

pain. Although the former usually subside with the onset of the cerebral reaction, the latter often continue as a part of it.

The approximate order of the mental confusion observed in slowly developing allergic cerebral syndromes consists of a shortening of the attention span, incomprehension in reading, sluggishness of association, impairment of recent memory, unresponsiveness to suggestion leading to negativism, inability to make decisions, crying, and various degrees of slowness of thought and speech. Aphasia is manifested by the partial or complete inability to express thoughts, groping for words, stuttering, or the use of expressions utterly out of context. Partial disorientation as to time and place is common, and experience indicates that patients in such a dazed state should be accompanied home.

More rapidly advancing cerebral reactions may begin with cessation of reading or with a relative detachment as if apprehensive or preoccupied. Then with little or no warning the subject may suddenly start sobbing, continuing with intermittent waves of crying and self-pity alternating with progressively longer periods of semistuporous sleep.

With more continuous and deeper stupor the subject is unable to move or speak but usually retains auditory perception. Although he may be partially aroused from this unrestful, disagreeable semisleep, it will end spontaneously by passing through a gradually diminishing twilight zone of extreme drowsiness, mental confusion and exhaustion, sometimes including headache and muscle trembling.

Advanced stages of this clinical picture in which impairment of cerebration predominates are often accompanied by varying degrees of altered cerebral motor function.

107. EXPERIMENTALLY INDUCED STUPOROUS DRUNKENNESS (SOMNOLENTIA) FOLLOWING THE TEST FEEDING OF ALLERGENIC FOODS OR EXPOSURE TO CERTAIN PETROLEUM PRODUCTS

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Somnolentia, defined in medical dictionaries as sleep-drunkenness, aptly describes certain cerebral allergic reactions characterized by varying degrees of muscular incoordination, hyperactivity, and stupor—a combination noted in the histories of 15 nonalcoholic patients and induced experimentally in 10 of them following the ingestion of allergenic foods or the ingestion or inhalation of certain nonmedicinal products of petroleum or coal-tar.

This clinical reaction in which impaired coordination and hyperactivity predominate appears to be more advanced than the cerebral syndrome without abnormal motor phenomena. Although these phases often alternate, the history may be dominated by one or the other for an appreciable period and stupor may occur in either.

When experimental somnolentia develops slowly, the following successive stages have been observed: Alternating initial waves of yawning or drowsiness and bouts of rhinitis, coughing, wheezing, or pruritus are gradually superseded by headache and a happy, carefree attitude and behavior not dissimilar to that of early alcoholic inebriation. Incoordination increases; euphoria if present is replaced by progressive mental haziness, drowsiness, and "sleep." Headache, muscular incoordination, and depressed spirits are apt to be accentuated on awakening.

In more rapidly developing episodes localized allergic symptoms are less evident, being overshadowed by an overwhelming sense of being doped or drugged and progressive restlessness, tenseness, impairment of the finer coordinated movements of the hands, difficulty in focusing, dysarthria, floating sensations, general clumsiness, and ataxic locomotion. Stupor may intervene suddenly and at any stage, sometimes without warning. The subject occasionally falls. Involuntary fibrillatory to coarse muscle contractions may occur, at times epileptiform in type.

Alternating waves of a stuporous sleep and hyperactivity may persist for variable periods. Stupor predominates in some but in others it is superseded by wavelike advances of partial to complete disorientation, muscular cramps, and uncontrolled movements. As in cerebral reactions without hyperactivity, the stupor is usually followed by partial to complete amnesia or aphasia. Excessive thirst, cephalalgia and fatigue, tenseness, jitteriness, insomnia, and muscle soreness often occur as residua.

Prognosis is good providing a specific allergic diagnosis is made and incriminated causative agents are avoided, but most cases are not investigated from this standpoint.

108. THROMBIN ACCELERATOR: A CLOTTING FACTOR OF PLASMA DEFICIENT IN CIRRHOSIS OF THE LIVER, MULTIPLE MYELOMA, AND ECLAMPSIA

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The thrombin time, that is, the clotting time of a mixture of oxalated plasma and bovine thrombin, is prolonged in a wide variety of diseases, including cirrhosis of the liver, multiple myeloma, and eclampsia. The prolonged thrombin time of such pathologic plasmas was shortened by the addition of an equal volume of fresh normal plasma but was still longer than normal. The difference between normal and pathologic plasmas was reduced by the addition of bivalent cations.

The prolonged thrombin time in the abnormal plasmas did not appear to be due to a circulating anticoagulant since none was demonstrable by the usual techniques. Neither was it the result of an alteration in the fibrinogen in the pathologic plasmas. When normal and abnormal plasmas were defibrinated with thrombin, the thrombin time of the abnormal plasma was still prolonged relative to the normal when bovine fibrinogen and thrombin were added to each.

Evidence was obtained which suggests that the prolonged thrombin time was the result of the absence of a constituent of normal plasma. When normal oxalated plasma was incubated for some hours at 37° C., there was a progressive prolongation of the thrombin time, although no appreciable change occurred in the same plasma at 4° C. Moreover, normal plasma stored at 4° C. or −20° C. for several months had a prolonged thrombin time compared to fresh plasma. Such stored plasma no longer shortened the prolonged thrombin time of pathologic plasma. Adsorption of fresh normal plasma with barium sulfate caused little or no prolongation of the thrombin time.

Thus it would appear that plasma may contain a labile factor which accelerates the conversion of fibrinogen to fibrin by thrombin. This factor may tentatively be called "thrombin accelerator," though there are as yet no data concerning its mode of action. This accelerator is apparently not required for clotting, but does shorten the clotting time. A deficiency of

thrombin accelerator is often the only abnormality detectable in the plasma of patients whose clotting times are prolonged. For this reason it is believed that thrombin accelerator deficiency may be one of the causes of a hemorrhagic state.

109. EFFECT OF VITAMIN B_{12} AND INTRINSIC FACTOR ON MATURATION OF MEGALOBLASTS IN TISSUE CULTURE

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(INTRODUCED BY OSCAR D. RATNOFF, M.D.)

Marrow aspirations from patients with pernicious anemia in relapse grow readily in tissue culture on glass surfaces or chick plasma clots. This growth is poor in serum from relapsed pernicious anemia patients but vigorous when B_{12} is added to the culture medium and accompanied by an increase of hemoglobinated erythroblasts.

By Lajtha's modification of the Osgood technique 10 megaloblastic marrows from pernicious anemia patients were cultured from 1 to 4 (usually 2) days in a medium of pernicious anemia serum alone, plus physiologic concentrations of vitamin B_{12}, normal gastric juice or intrinsic factor concentrate, intrinsic factor source and B_{12} together, and, in some instances, folinic or folic acid. The percentages of megaloblasts and normoblasts in smears of each culture were compared with a control. Four megaloblastic marrows from patients with sprue and a megaloblastic hemolytic anemia (both of whom responded clinically to B_{12}) were similarly cultured.

Maturation occurred in all of the control specimens at a variable speed and was inconstantly affected by the addition of B_{12}, folinic acid, or intrinsic factor alone. In 11 of the 14 cultures B_{12} plus intrinsic factor showed less maturing effect than B_{12} alone. In no instance did it show more. In only a few instances were the differences between controls and experimental counts statistically significant. However, the chance likelihood of the trend shown in the B_{12} plus intrinsic factor cultures appears small. We conclude that the suspension technique is a difficult one with which to obtain clear-cut results.

We have been unable to confirm the claims of others that intrinsic factor enhances the megaloblast maturing effect of B_{12}. From these observations it appears that, as with bacterial cells, intrinsic factor makes B_{12} less available for use by growing red cells in vitro.

110. THE SERUM IRON IN EXPERIMENTAL HEPATIC INJURY

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(INTRODUCED BY SLOAN J. WILSON, M.D.)

Significant elevations of serum iron have been reported in infectious hepatitis. Therefore, the effect upon the serum iron in experimental hepatic injury of several etiological and anatomical types was studied. The dogs were sacrificed at various intervals during the course of hepatic injury and data were obtained (a) on the qualitative and time relationship between hepatic

injury and serum iron changes and (b) on the quantitative relationship between the magnitude of serum iron changes and the extent of hepatocellular necrosis.

Chemotoxic centrilobular necrosis induced by oral carbon tetrachloride produced significant hypersideremia in all of 24 dogs. The rise in serum iron coincided with the appearance of hepatocellular necrosis, beginning at eighteen hours and reaching a maximum on the third or fourth day. There was a good correlation between the per cent of necrosis of parenchymal tissue and the serum iron elevation.

Sublethal doses of allyl formate intraperitoneally produced less pronounced serum iron increases and the histologic examination revealed only moderate periportal necrosis.

Ischemic necrosis due to hepatic artery ligation produced significant hypersideremia during the fourth through the tenth postoperative days, in all of nine dogs where the massive anaerobic bacterial necrosis was prevented by means of penicillin. Five animals without penicillin died within forty-eight hours and no iron increases were seen.

Inoculation with virus of canine hepatitis produced hypersideremia only in a small number of dogs where typical hepatocellular necrosis developed. In the majority of 26 dogs the so-called canine hepatitis was found to be a generalized viral infection with little hepatocellular damage.

Ligation of the common duct produced in most animals a moderate hypersideremia about the tenth day and a second gradual rise beginning at the end of the third week. These findings will be discussed in connection with the corresponding anatomical changes.

Surgical removal of 60 per cent of liver tissue did not change the serum iron level, suggesting that the absence of storage tissue is not a factor in the development of hypersideremia in liver disease.

The common denominator in the observed serum iron increases was acute hepatocellular necrosis. The possible underlying mechanism will be discussed.

111. A BETTER METHOD TO OBTAIN SAGITTAL AND OTHER COMPONENTS OF THE MEAN CARDIAC VECTOR AND ITS IMPORTANCE IN VECTORCARDIOGRAPHY

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The "lead vector" of Burger and van Milaan and the closely related but simpler concepts of the "lead field" proposed by McFee and the "tubes of influence" of Lepeschkin provide the theoretical basis for the development of new leads which should give records that represent the transverse, sagittal, and vertical components of the *mean* cardiac vector more exactly than is true of the leads now being employed for this purpose.

For example, the lead system for obtaining the true sagittal component of the mean cardiac vector is associated with a lead field that can be represented by uniformly spaced parallel lines passing in an anterior-posterior direction through the entire heart. The reciprocity theorem tells us that a grid arrangement with many small electrodes located on the precordium and on the posterior chest wall behind the heart will give a lead field of this type.

Examination of the lead fields associated with the cube system originally advocated by Duchosal and Sulzer and extensively used by Grishman and associates makes it clear that this is a poor method to obtain the transverse

and vertical as well as the sagittal component of the mean cardiac vector. A similar scrutiny of the lead fields that exist with the system that uses the equilateral tetrahedron indicates that, while the transverse and vertical components are fairly accurate, the sagittal component is subject to rather large errors.

In this paper, figures illustrating the above matters are presented and actual records, including vectorcardiograms, taken by the different methods are shown.

Additional studies indicate that the use of a large number of small electrodes in the grid system is not necessary except when the grid is located close to the heart. The use of flexible metal plates or a metal mesh to replace the multiple small electrodes in the grid is being investigated.

It is concluded that current methods for taking vectorcardiograms, especially the cube system, are subject to large errors and a better technique employing grids with multiple small electrodes is proposed.

112. EFFECT OF PREOPERATIVE TREATMENT WITH PENICILLIN ON BACTERIAL CONTENT OF EXCISED TONSILS AND ON POSTTONSILLECTOMY BACTEREMIA

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WITH THE TECHNICAL ASSISTANCE OF DORIS ADAIR (BY INVITATION)

In a control series of 69 patients who had no antibiotic treatment immediately preceding tonsillectomy, 57.4 per cent had beta hemolytic streptococci in quantitative cultures of the excised tonsils, although these organisms were found in only 33⅓ per cent of throat cultures taken just before tonsillectomy.

Among those patients receiving penicillin in doses averaging 600,000 units the day before tonsillectomy or for several days before tonsillectomy, 20 per cent had beta hemolytic streptococci in the excised tonsils, but these organisms were found in none of the throat cultures taken just before operation.

In the "unprotected" group the average bacterial count was 476,000,000 per gram of tonsil for beta hemolytic streptococci, and 124,000,000 per gram for alpha and gamma streptococci. The bacterial count of the tonsils of persons receiving penicillin only one day before tonsillectomy and in many instances also one hour before tonsillectomy was 142,000 for beta hemolytic streptococci and 24,000,000 per gram for alpha and gamma streptococci. Among the persons receiving 600,000 to 800,000 units of penicillin daily for four to ten days prior to tonsillectomy, no beta hemolytic streptococci were found in quantitative cultures of the excised tonsils. The count for alpha and gamma streptococci was 18,000,000 and for staphylococci 112,000,000. In the latter group of thirteen cases, *E. coli* was the predominating organism in four, *Kl. pneumoniae* in one, *M. tetragenus* in one, *Staphylococcus albus* in one, and alpha and gamma streptococci in six. In this group the quantitative count for staphylococci was much higher than in the others.

Blood cultures taken immediately after tonsillectomy were positive in 31.7 per cent of the unprotected group and in 20 per cent of the group receiving penicillin preoperatively. The incidence of bacteremia in those receiving only one or two doses of penicillin preoperatively within the twenty four hours before operation was 29 per cent. There was only one positive blood culture (7.7 per cent) among the persons receiving penicillin daily for four to ten days before tonsillectomy. The microorganisms cultured from the

blood were gamma or alpha streptococci, seventeen times, beta hemolytic streptococci, four times, beta hemolytic and gamma streptococci together, one time, and pneumococci, three times.

113. SPONTANEOUS DIABETES IN DOGS: AN ACCOUNT OF EIGHT CASES

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The cases here described were referred by veterinarians because of polyuria, polydipsia, and glycosuria. Duration of symptoms was from two weeks to four years. Most of the dogs were aged, obese, female mongrels. Nearly all had advanced cataracts. Initial blood sugars ranged from 237 to 780 mg. per cent. All but one had heavy ketonuria and three were in frank acidosis. Hypercholesterolemia was the rule. Control of glycosuria was fair to poor with from 10 to 40 units of insulin daily.

Pathologic observations were made in six of the eight dogs. In all but one the pancreas was grossly normal, but the islands of Langerhans contained practically no recognizable beta cells. In three cases with diabetes of brief duration there was classical hydropic degeneration and in one of these glycogen was found within the islets. Arteriosclerosis was present in five cases, but is also found in nondiabetic old dogs. Abnormalities of the glomeruli, possibly nonspecific, were seen in five instances. In one dog with glomerular lipidosis the lesions closely resembled, but perhaps were not identical with, the spheroid bodies of intercapillary glomerulosclerosis in man. Such lesions have not been found in nondiabetic old dogs or in any of fourteen autopsied dogs with experimental diabetes of from one to three years' duration.

In the four animals in which it was determined, the insulin content of the pancreas (Dr. Wrenshall) was far below normal.

These studies, together with others in the literature, establish the fact that spontaneous canine diabetes is characterized by a demonstrable organic defect of the beta cells. The finding of hydropic degeneration and glycogen infiltration of the islets in cases of recent onset supports the concept that this is an early, specific lesion causally related to the disease and having general biologic significance. If further observations confirm the occurrence of a Kimmelstiel-Wilson type of lesion in dogs with spontaneous diabetes, and its absence in experimental animals, it would suggest that the naturally occurring disease carries with it a tendency to this kind of glomerular damage which hyperglycemia and glycosuria, by and of themselves, do not produce.

114. EFFECT OF CORTICOTROPIN ON CELL FORMATION IN THE BONE MARROW, SPLEEN, AND THYMUS OF THE RAT

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The effect of corticotropin in reducing the leukocyte count in acute leukemia is well known. In two cases of subacute lymphocytic leukemia the peripheral leukocyte count fell to an average of 4 per cent of the pretreatment level after six days of corticotropin therapy. The depressive effect of corticotropin on lymphoid tissue in animals has been attributed to lymphocytolysis and mitosis inhibition.

In the present investigation serial changes in cellularity, mitosis rate, and total new cell formation in the bone marrow, spleen, and thymus of the rat receiving 6 to 8 units of corticotropin daily for periods of three, ten, and twenty days were studied. Indices of these changes were obtained from estimates of the phosphorus content and P^{32} incorporation into the DNA fraction of these organs.

Total new cell formation and cellularity per organ declined progressively in the bone marrow, spleen, and thymus throughout the twenty-day period. In the bone marrow there was a gradual decrease in total new cell formation to 75 per cent of normal and the cellularity showed a similar decline. The fact that there was no significant change in the per cent of dividing cells (specific activity) suggests that in addition to mitosis inhibition, there was a slight acceleration of cell maturation and delivery. Total new cell formation in the spleen was reduced to 15 per cent of normal with the greater proportion of the fall occurring in the last ten days. Because the decline in cellularity lagged behind the decline in total new cell formation, it appears that mitosis inhibition was a major factor in splenic involution. In the thymus, total new cell formation decreased to a plateau level of approximately 15 per cent by the third day. Mitosis inhibition was important in the initial reduction in the size of the thymus.

This study shows the differential effect of corticotropin on the organs studied and throws some light on the mechanism of its action in acute leukemia.

115. STUDIES ON THE MECHANISM OF MURMUR PRODUCTION

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Murmurs are commonly ascribed to Reynolds turbulence and vortex formation in the blood stream. However, the vibration frequencies produced by such nonlinear flow are very much higher than those heard clinically. Our studies have suggested that murmurs result from the special properties of flow through collapsible vessels or orifices, as through arteries or valves. Such flow produces no sounds unless the lumen is critically narrowed, producing flow at high velocities. At critical degrees of narrowing, the intraluminal pressure drops markedly because of the high velocity of the stream (Bernoulli); the higher extraluminal pressure causes the vessel or orifice to close suddenly and stops the flow. At this point, all the energy of the stream is converted into pressure and the collapsed lumen is forced open; flow starts again and soon reaches the critical velocity for reclosing. These cycles have been studied with cinematography at 4,000 frames/second and by special sound analysis techniques. The repetition rate of this phenomenon has been shown to depend on three major physical factors: driving pressure head, orifice (diameter, length, and stiffness), and pressure beyond the opening. Other factors being equal, no murmurs are produced when the lumen is strongly compressed. As the degree of compression is reduced, high-pitched murmurs (e.g., 300 repetitions per second) are produced. Further decompression of the orifice results in a reduction in repetition rate to 10 per second or even less. At a critical lumen diameter the cycle stops and no murmurs are heard. These studies demonstrate that either extreme stenosis or a wide orifice results in no murmur production; the repetition rate (pitch of the murmur) increases with the degree of stenosis; loudness correlates with volume flow through the opening.

116. AN ANALYSIS OF THE RESPIRATORY FLUCTUATIONS IN PULMONARY ARTERIAL PRESSURE TRACINGS IN MAN

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A survey of the characteristics of respiratory organs suggested that much of the regulation of the pulmonary circulation resided in the bronchomotor apparatus (Am. J. Med., in press). Increased bronchomotor tone was thus interpreted as a means of entrapping air in the alveoli during expiration, acting as a built-in positive-pressure mechanism to counteract tendencies to pulmonary congestion and edema. When transient emotional episodes (anxiety, crying) are excluded, fluctuations in the pulmonary arterial pressure reflect respiratory effort and intrathoracic pressure changes, and may provide an indirect assay of bronchomotor tone.

To test this thesis, the respiratory variations in the pulmonary arterial pressure tracings obtained during catheterization in 73 patients were analyzed. In 6 patients without clinical cardiopulmonary disease, the fluctuations ranged from 5 to 8 mm. Hg systolic, and 4 to 6 mm. Hg diastolic. The same degree of fluctuation was obtained when the pulmonary arterial pressure was in the normal range in mitral stenosis (13 cases) and systemic hypertension (1 case). In chronic cor pulmonale, 4 of 6 patients with a normal pulmonary arterial pressure had an enhanced sweep (10/5 mm. Hg); all 4 with pulmonary hypertension had markedly enhanced fluctuations (17/10 mm. Hg). In pulmonary hypertension subsequent to mitral stenosis (7 cases), or systemic hypertension (4 cases), the excursions were marked (14/13 mm. Hg mean). Associated with the heightened fluctuations there was a prolongation of the heightened pressure phase (expiration) compared with the inspiratory phase. By contrast, in Eisenmenger's complex (6 cases) and in pulmonic stenosis (8 cases) there were virtually no respiratory fluctuations in the pulmonary arterial pressures (2/2 mm. Hg).

These data show that pulmonary air entrapment (emphysema, chronic cor pulmonale) produces an enhanced respiratory sweep in the pulmonary arterial pressure. In addition they suggest that in conditions tending to pulmonary capillary congestion and edema (mitral stenosis, systemic hypertension) bronchomotor tone is augmented, air is trapped in expiration, prolonging and heightening the fluctuations. It is significant that when precapillary resistance is augmented, pulmonary capillary congestion is absent and bronchomotor mechanisms are not invoked; respiratory fluctuations are therefore minimal even in the face of marked pulmonary arterial hypertension (Eisenmenger's complex).

117. TIME-LAPSE MICROCINEMATOGRAPHIC STUDIES OF THE L. E. PHENOMENON

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(INTRODUCED BY PAUL J. FOUTS, M.D.)

We have previously described our concepts of the formation of the L. E. cell on examination of supravitally stained preparations. It was our concept that the L. E. factor acted in two ways: (1) it initiated premature nuclear autolysis of polymorphonuclear leukocytes which became L. E. bodies, and (2) it promoted phagocytosis of these lysed granulocytes by intact neutrophilic, eosinophilic, and basophilic leukocytes.

This concept is confirmed by time-lapse micro movies of supravitally stained bone marrow preparations. In these sequential studies formation of rosettes, "signet rings," and clusters typical of this phenomenon are illustrated. These studies illustrate the additional fact that phagocytosis is not complete until at least partial dissolution of the cytoplasmic membrane is presented to the phagocytic leukocyte.

118. STUDIES ON THE MOTILITY PATTERNS OF LYMPHOCYTES

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(INTRODUCED BY PAUL J. FOUTS, M.D.)

The morphologic characteristics of lymphocytes in a variety of infectious and neoplastic diseases have been the subject of considerable controversy. Attention has been focused on chromatin patterns, quantity of cytoplasm, mitochondrial activity, and granular prominence, but, to date, little attention has been accorded the motility of these cells.

Present-day concepts would suggest that there are (a) normal lymphocytes, (b) abnormal lymphocytes which are characteristic of infectious mononucleosis although found also in other viral infections, and (c) abnormal lymphocytes peculiar to (1) acute and (2) chronic lymphocytic leukemias.

In our experience all standard staining techniques interfere with the study of these cells, chiefly by their retardation of cell motility. We have found that the static appearance of stained lymphocytes might lead to erroneous concepts which must be altered after examination of fresh unstained preparations.

It is our observation that the static morphologic pattern of lymphocytes is a result of two factors: (a) maturity and (b) motility. The study of the cellular maturity of lymphocytes is not dependent upon the staining technique used. The use of time-lapse micro movies of unstained lymphocytes, however, has enabled us to study in more precise detail the characteristic motility of lymphocytes in normal individuals and in patients with various disease entities in which there are described lymphocytic abnormalities. From these studies it can be seen that in many cases the characteristic morphologic appearance of a lymphocyte often depends upon its state of motility at any given instant, and that often the same cellular morphology may be seen in different disease states in which lymphocyte motility is similar.

The characteristic motility of lymphocytes from normal individuals and from patients with infectious mononucleosis, pernicious anemia, and acute and chronic lymphocytic leukemia is clearly illustrated in a series of time-lapse micro movies employing supravital and unstained preparations with standard and dark phase illumination.

119. THE ERYTHROPHAGOCYTIC PROPERTIES OF SERUM FROM PATIENTS WITH ACUTE DISSEMINATED LUPUS ERYTHEMATOSUS

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(INTRODUCED BY PAUL J. FOUTS, M.D.)

Many observers have noted that when L. E. serum is incubated with donor leukocytes from another individual, L. E. cells will be formed. Under certain conditions, however, it has been our experience that this phenomenon is overshadowed or even completely blocked by the development of active erythrophagocytosis.

Our first observation of this phenomenon was made when the serum from a patient with known acute disseminated lupus erythematosus was incubated with leukocytes obtained from a patient with congenital hemolytic anemia. On examination of this preparation it was noted that, while leukocytes contained one to many phagocytized erythrocytes and peculiar "clear bodies," careful search failed to reveal a single clearly defined L. E. cell. This phenomenon was then checked by employing known L. E. serum against donor leukocytes from normal individuals and from a considerable number of patients with a variety of disease entities.

When known L. E. serum was incubated with leukocytes from patients with primary and secondary hemolytic anemia, Hodgkin's disease, polycythemia rubra vera, chronic lymphocytic leukemia, leukolymphosarcoma (acute lymphatic leukemia), Gaucher's disease, reticulum cell sarcoma, follicular lymphoma, sarcoidosis, and sickle cell disease, erythrophagocytosis strikingly predominated over formation of L. E. cells.

When, however, known L. E. serum was incubated with donor leukocytes from normal controls, acute bacterial infections, carcinomas (both untreated and irradiated), and thrombocytopenic purpuras, formation of L. E. cells was usual and erythrophagocytosis was rarely observed.

In those preparations in which L. E. serum was incubated with donor leukocytes from patients with chronic myelogenous leukemia, L. E. cell formation and erythrophagocytosis were encountered with equal frequency.

Illustrative graphs and photomicrographs demonstrating this phenomenon are presented and some speculation as to the cause of this phenomenon and the precursor of the "clear body" is presented.

120. ESSENTIAL TELANGIECTASIA, PULMONIC AND TRICUSPID STENOSIS, AND NEOPLASTIC LIVER DISEASE. A POSSIBLE NEW CLINICAL SYNDROME

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Two patients who were observed over a period of several years until their death presented an unusual combination of clinical and pathologic manifestations such that the possibility occurs that a hitherto unreported clinical syndrome may have been encountered. The second case resembled the first so closely that the correct diagnosis was made ante mortem. The initial and most striking clinical feature was a profound telangiectasis appearing first on the face, later extending to the thorax, abdomen, and upper extremities, and ultimately becoming so severe that the face took on a frightening, purple-gray hue. The first evidence of telangiectasis preceded death by twenty years in one patient and by ten years in the other. Histologic studies of the skin from the involved areas disclosed greatly dilated veins considered to be pre-existing rather than newly formed veins and capillaries.

The later years of life were characterized by progressive congestive heart failure, primarily right ventricular in origin. Post-mortem examination disclosed severe pulmonic and tricuspid stenosis with minor mitral valvular involvement in each patient. The valvular disease was considered of rheumatic origin, although Aschoff bodies were found in only one case. Hepatomegaly preceded death by ten years in one patient and two years in the other. There were extensive hepatic metastases from a carcinoid of the ileum in the former and an extensive adenocarcinoma of the hepatic bile ducts in the latter.

The venous pressures which were recorded in these patients did not appear of sufficient magnitude to produce this type of dilatation of the capillaries and venules. The telangiectasis was of such great duration that in all probability it antedated the hepatic disease. The telangiectasis therefore remains unexplained although it may have been the end result of some peculiar capillary damage due to the initial rheumatic disease which also produced the unusual valvular disturbance.

121. THE STOMACH IN TREATED PERNICIOUS ANEMIA: A CYTOLOGIC STUDY

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(INTRODUCED BY JOSEPH B. KIRSNER, M.D.)

Pernicious anemia is one manifestation of a systemic disease caused by a nutritional deficiency secondary to an abnormal gastric mucosa. Replacement therapy corrects the metabolic defect in the nervous system and the blood; however, the gastric changes are irreversible, as indicated clinically by the persistence of achlorhydria and the increased incidence of gastric carcinoma and polyposis. Gastric biopsy observations in our laboratory also confirm several pathologic studies indicating the permanence of gastric glandular atrophy.

The present study demonstrates a characteristic appearance of the exfoliated cellular material from the stomach in patients with pernicious anemia, persisting despite prolonged therapy. "Chymotrypsin lavage" and the "antrol abrasive balloon" provided excellent cytologic material. Two types of atypical columnar cells were demonstrated. Type I cells were enlarged and contained nuclei approximately twice normal size, varying from a round, bland to an irregular, hyperchromatic, almost anaplastic appearance. Type II columnar cells were slender and elongated, containing either small pyknotic or large vesicular nuclei, centrally located in the cell. Various gradations existed between these two extremes. Type I cells seemed specific for pernicious anemia and were demonstrable in 20 of 21 patients who had been adequately treated from 1.3 to 23 years (average, 7.5 years). Untreated patients with pernicious anemia also were examined for comparison.

Type I cells were present occasionally in smears prepared from multiple scrapings of freshly resected pernicious anemia stomachs. The source of these cells was not apparent in long strips of gastric mucosa examined histologically by the "Swiss roll" technique.

Further investigation is necessary to determine whether this cytologic picture is pathognomonic for pernicious anemia. If it proves to be specific, it will permit the diagnosis despite the institution of therapy. An understanding of the benign cytology in pernicious anemia is essential in avoiding false diagnoses of malignancy and in facilitating the detection of gastric carcinoma, a frequent complication of this disease.

122. ACETAZOLEAMIDE STUDIES IN CARDIAC EDEMA

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(Introduced by W. C. Levin, M.D.)

Acetazoleamide (Diamox, carbonic anhydrase inhibitor) was administered in 27 cases of heart failure. In 12 patients, 500 mg. was given in one dose by mouth at 6 A.M. In 15, 500 mg. (or more) was administered every six hours for 4 doses. In 3 instances both methods were used at least five days apart for comparative purposes.

Followed closely, daily or every six hours were: serum sodium, potassium, nonprotein nitrogen; urine volume, sodium, potassium; body weight; clinical state.

Diuresis was achieved (50 per cent to 600 per cent 24-hour urinary output increase) in 22 of the 27 cases. Successful diuresis (up to 600 per cent 24-hour urinary output increase) was noted in 3 patients previously refractory to mercurial diuretics. Water excretion was accelerated not only in the first six hours, as in normal dogs (T. H. Maren), but also during the next few days. Corresponding weight losses of 2 to 5 pounds per day in successful cases, and up to 22 pounds in fourteen days (e.g., after 2 Gm. course), were noted. Immediate diuresis and weight loss were approximately equal after single or multiple doses. Multiple doses, however, seemed to prolong both of them.

Clinical remission of dyspnea and congestive failure was the rule. Toxic psychosis was seen once—in uremia. Otherwise, anorexia, nausea, vomiting, malaise—in 3 cases (from 2 to 4 Gm. per day). The urine sodium also rose in the first six hours and for as long as five days (maximum followed). The serum sodium was unchanged. Potassium excretion was accelerated especially after multiple doses and was often delayed. Serum potassium usually fell after multiple large doses to as low as 3.8 meq./l.

Further clinical and enzymatic-chemical studies are in progress.

123. A STUDY OF PULSUS ALTERNANS

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(Introduced by B. K. Wiseman, M.D.)

During an investigation of pulsus alternans it was found that this phenomenon could at times be rather profoundly influenced by changes in the venous return to the heart.

Twenty patients with organic heart disease characterized by left ventricular enlargement form the basis of this study. The direct arterial pulse, heart sounds, and electrocardiogram were recorded simultaneously.

Only two patients had established alternans. Exercise and passive leg raising lessened the degree of alternans in these subjects, whereas venous tourniquets and sudden tilting to the upright position exaggerated this phenomenon. Pulmonary arterial pressures recorded simultaneously with the peripheral pulse in one of these subjects showed this phenomenon to be limited to the left side of the circulation. In only one of 18 patients did the stress of exercise bring out an alternans when it was absent at rest. Venous tourniquets also caused pulsus alternans in this patient, to an even greater degree than did exercise. The occurrence of short runs of alternation following a ventricular

premature beat was noted in six patients, the duration of this alternation being related to the state of the venous returns in the same manner as established alternans.

These observations suggest that decreasing the venous return to the heart will accentuate pulsus alternans and increasing this return will diminish it. They further suggest that pulsus alternans might be more easily detected clinically if the patient is upright.

124. THE ELASTICITY OF THE CRANIOSPINAL VENOUS BED

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CINCINNATI, OHIO

WITH THE TECHNICAL ASSISTANCE OF DORIS LAMB, B.S. (BY INVITATION), EDYTHE BARNES, B.A. (BY INVITATION), JOAN JACKSON, M.S., (BY INVITATION), BETTY WAYNE, B.S. (BY INVITATION), AND WILLIAM BROSENE, B.S. (BY INVITATION)

The pressure in the spinal subarachnoid fluid space or the lateral ventricles has been recorded continuously on several hundred patients, living and dead, with diverse disorders of the central nervous system. The effect on the pressure of adding fluid to, or withdrawing fluid from, these fluid spaces has been studied. The fluid pressure changes are such that the rate of change of pressure with respect to the volume of fluid added or withdrawn is a minimum when the fluid pressure is near the mean systemic venous pressure. The fluid pressure at which the minimal value of this derivative of pressure appears varies with the systemic venous pressure. The derivative increases symmetrically and progressively more rapidly as the fluid pressure is raised above or lowered below the systemic venous pressure, having the general form of a hyperbola. When the procedure is performed with an inflated cuff around the neck two minima appear. One depends on the venous pressure caudad to the cuff; the other on the venous pressure craniad to the cuff. The parameters of the function with the caudad minimum are similar to those of isolated spinal curves. The procedure without the cuff also induces the seepage of fluid in the direction of returning the pressure to its original value. Skull fractures and defects have little effect on the parameters. Methods of estimating these parameters and the errors of these estimates will be described.

From these observations we have inferred that the changes in cerebrospinal fluid volume induced by the procedure lead to an equal and opposite change in the volume of the craniospinal venous bed rather than to an appreciable change in the total volume of the craniospinal contents. Therefore, this pressure-volume curve reflects the elasticity of the craniospinal venous bed and not that of the skull or the craniospinal limiting membranes. This venous bed becomes more rigid under certain clinical conditions, notably those characterized by brain swelling, and less rigid than normal in other states, such as cerebral atrophy. Localized space-occupying lesions such as tumors, hemorrhages, and infarcted areas, when they are moderate in extent, have much less of an effect on this function.

125. PREOPERATIVE, SIX-WEEK AND TWELVE-MONTH POST-OPERATIVE HEMODYNAMIC STUDIES IN PATIENTS UNDERGOING MITRAL COMMISSUROTOMY

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(Introduced by Roy W. Scott, M.D.)

Eleven patients have been studied by cardiac catheterization preoperatively and six weeks and twelve months following mitral commissurotomy. In all instances the diagnosis of mitral stenosis was clinically well established and symptoms had been present for several years. MVA was 1.2 cm.^{2} or less (surgeon's estimate), and in no instance was it possible to demonstrate surgically induced mitral insufficiency.

In all subjects objective improvement (reduction in pulmonary arterial and "pulmonary capillary" pressures and in calculated pulmonary resistances) was noted six weeks postoperatively, as well as subjective clinical improvement. In 7 patients with "pure" mitral stenosis, the one year postcommissurotomy studies demonstrated maintenance and/or further improvement over the six-week values. In 4 with associated lesions (coronary artery sclerosis, 1, mitral and aortic insufficiency, 1, aortic stenosis, 1, mitral insufficiency, 1), regression toward the preoperative values was observed, although subjective improvement noted following commissurotomy was maintained. This may be partially explained by the ability to raise the cardiac output under stress to a greater extent than was possible preoperatively. Values for walking ventilation and maximal breathing capacity reasonably paralleled the hemodynamic changes, although MBC was often transiently reduced (6 weeks) in association with pleurodiaphragmatic dysfunctions secondary to surgical interference.

The findings to date suggest that the best long-term results are to be expected in patients with mitral stenosis unaccompanied by other cardiac lesions.

126. P VECTOR LOOP IN NORMALS, IN RIGHT AND IN LEFT ATRIAL HYPERTROPHY, AS STUDIED BY THE TECHNIQUE OF ELECTRICAL DISSECTION OF THE VECTORCARDIOGRAM (DIFFERENTIAL VECTORCARDIOGRAPHY)

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Eugene Vayda, M.D. (By Invitation)
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Because of difficulties in distinguishing the individual characteristics of the component loops of the vectorcardiogram as obtained with the cathode ray oscilloscope, a systematic study of the P loop has previously not been possible. The development of a practical apparatus to dissect the vectorcardiogram (Hellerstein, Shaw, and Sano) has made it possible to study the fine details of the component loops of the vectorcardiogram.

The isolated P vector loops were obtained by the technique of electrical dissection in 69 cases: 12 normals, 16 cases with practically normal P waves, 20 cases with left atrial hypertrophy, 3 cases with borderline left atrial hypertrophy, 13 cases of right atrial hypertrophy, 2 cases of combined atrial hypertrophy, and 3 cases with ectopic origin of the P wave.

Exclusive of the P vector loops of ectopic origin, the direction of inscription of the P vector loop in most cases is counterclockwise in the frontal

and horizontal planes, and clockwise in the sagittal plane. The normal P loop is small, closed, and directed almost vertically inferiorly. The P loop of left atrial hypertrophy is larger, open, and directed posteriorly, inferiorly, and leftward. That of right atrial hypertrophy is the largest, usually open, and directed anteriorly and inferiorly. The morphology of the P vector loop can readily be related to the anatomic location of the hypertrophied atrium. The vector P loop is often helpful clinically in the differentiation between right and left atrial hypertrophy when routine electrocardiograms are equivocal.

127. THE RELIEF OF RESISTANT EDEMA BY UTILIZATION OF A SUMP PHENOMENON

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Twelve cases of massive *peripheral* edema with hydrothorax and/or ascites have been resistant to usual methods, but have been relieved by successive aspirations of fluid from either the pleural or peritoneal spaces. The repeated aspirations did not lower the serum sodium or albumin levels; nor were they followed by post-aspiration diureses.

From 40 to 90 per cent of the sodium and water eliminated, to achieve a "dry-weight," was removed mechanically, rather than coaxed out via reluctant kidneys. In some instances dry weight was achieved with a weight loss which corresponded very closely to the weight of the aspirated fluid: (F. S. 14.0 kg., 15.2 L. via the thorax; R. W. 22.0 kg., 22.0 L. via the abdomen). In the intervals *between* aspirations, the signs of fluid in the chest or abdomen *increased* as the signs of peripheral edema *decreased* without any change in total body weight. The fluid of the interstitial space and of the untapped space appeared to seep readily into the "sump" from which the fluid had just been removed.

In most instances after dry weight was reached the usual regime, which had been enforced from the beginning but had proved ineffective, was effective in preventing reaccumulations. However, in some cases it was observed that in order to anticipate or prevent a recurrence of peripheral edema and either hydrothorax or ascites, it was necessary to aspirate the "sump" at intervals for some months. Failure to do so after 500 ml. to 1,500 ml. of fluid had collected in the "sump" was followed by the reappearance of generalized edema, suggesting that seepage from the "sump" was occurring in the opposite direction.

The advantages of the recognition of this phenomenon are obvious in those cases in which altered hemodynamics, or intrinsic impairment of the kidneys, or an obscure endocrine disturbance are preventing progress in spite of proper attention to the usual regime. As a means of by-passing reluctant kidneys the utilization of this "sump" phenomenon has proved simple, safe and effective clinically in some very obstinate cases.

128. A NEW TEST FOR INTRINSIC FACTOR ACTIVITY

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(Introduced by Ovid O. Meyer, M.D.)

Normal human gastric juice contains a substance, intrinsic factor, which enhances the oral hematopoietic activity of vitamin B_{12} in patients with pernicious anemia in relapse. The most likely mechanism through which in-

trinsic factor works is in the transport of vitamin B_{12} across the intestinal mucosa. Progress toward the isolation of this substance has been hampered by the necessity of a tedious bioassay in patients with pernicious anemia in relapse.

Each of six normal persons was given 2 μg of radioactive B_{12} by mouth and two hours later each was given 1,000 μg of nonradioactive B_{12} by subcutaneous injection. Beginning at about four to six hours after the oral dose, radioactivity appeared in the urine for at least twenty hours. Ten patients with known pernicious anemia but in therapeutic remission were treated as were the normal, and none of them excreted radioactivity in the urine in the next twenty-four hours. When these same pernicious anemia patients were given 25 to 100 ml. of neutralized normal human gastric juice with the 2 μg of radioactive B_{12}, followed in two hours by the "flushing" injection of nonradioactive B_{12}, each of them excreted easily detectable radioactivity in the urine. Boiling the gastric juice, before or after adding the radioactive B_{12} to it, destroys its ability to cause radioactivity to appear in the urine of pernicious anemia patients. If active gastric juice is given twelve hours after the oral $B_{12}Co^{60}$ and ten hours after the subcutaneous injection of nonradioactive B_{12}, no radioactivity was found in the urine excreted during the thirty-six hours following the oral $B_{12}Co^{60}$. Two preparations of hog duodenum known to be active as intrinsic factor when tested clinically in pernicious anemia patients in relapse produced radioactivity in the urine of pernicious anemia patients when given simultaneously with the oral $B_{12}Co^{60}$.

This test can be done repeatedly on pernicious anemia patients who are in complete remission. The limiting factor is the safe dose of Co^{60}.

129. EXPERIMENTAL HEPATIC PORPHYRIA: EXCESSIVE PORPHYRIN FORMATION RESULTING FROM DISTURBANCE IN LIVER CATALASE METABOLISM

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(Introduced by C. J. Watson, M.D.)

In previous studies we have reported the production of a form of experimental porphyria in rabbits and rats by oral administration of Sedormid. The porphyrin was believed to originate in the liver, since only this organ exhibited great excess of proto-, copro-, and uro type-porphyrins and porphobilinogen, which were excreted partly in the bile and partly in the urine. The present report is concerned with the effect of Sedormid, in rats, upon the hepatic metabolism of two heme-proteins, catalase and cytochrome C.

Sedormid administration causes a rapid fall in liver catalase activity to values below 10 per cent of normal. This decline is associated with excessive porphyrin formation in the liver. Catalase activity is unaffected in other systems such as the circulating erythrocytes and the kidneys. Cytochrome C concentration per milligram of liver protein nitrogen remains normal, even after continued Sedormid administration for forty-two days. The hemoglobin and hematocrit in such rats do not change significantly.

To investigate the selective effect of Sedormid upon liver catalase, the rate of incorporation of glycine-2-C^{14} into the prosthetic heme-group of this enzyme was studied. Liver catalase preparations were checked for purity from contamination by other heme-compounds, by paper electrophoresis. The catalase hemin was converted to crystalline mesoporphyrin methylester for radioactive measurements. A significant difference in the rate of incorporation of radio-

active carbon was found between normal and Sedormid-treated animals, indicating an almost complete block in liver catalase synthesis in Sedormid-poisoning. The excessive formation of porphyrin by the liver may be related to an attempt to overcome this block. The effect of various substances on Sedormid porphyria will be discussed.

130. THE EFFECT OF ACTH ON THE METABOLISM AND EXCRETION OF ASCORBIC ACID

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(Introduced by Robert M. Kark, F.A.C.P., F.R.C.P.)

The effect of ACTH on the metabolism and renal excretion of ascorbic acid was studied in healthy individuals. For one week prior to the studies they took daily 1.0 Gm. of ascorbic acid by mouth in order to saturate their body stores. When this was accomplished, the plasma level was elevated by intravenous injection of ascorbic acid, using a standard infusion pump and the maximum reabsorbtive capacity of the renal tubules for ascorbic acid was calculated for both total plasma ascorbic acid and reduced ascorbic acid. Plasma ascorbic acid was determined by two methods; the Roe and Kuether technique was used to determine total ascorbic acid and indol-phenol was used to titrate reduced ascorbic acid. Glomerular filtration rate and effective renal plasma flow were determined from the clearances of inulin and para-amino-hippurate, respectively. Following completion of control studies, 100 mg. ACTH-gel was administered intramuscularly in divided doses over thirty-six hours. Four hours after the last injection of ACTH, all studies were repeated. At this time absolute eosinophil counts confirmed the presence of an ACTH effect. The results revealed no effect of ACTH on the level of total plasma ascorbic acid but the amount of ascorbic acid present in the plasma in the reduced form increased during the administration of ACTH. In two representative studies the percentage of reduced ascorbic acid in the plasma rose from 54 per cent to 65 per cent and from 62 per cent to 81 per cent, respectively. On the other hand, there was no significant change in the percentage of reduced ascorbic acid in the urine in these subjects. Only small changes in glomerular filtration rate and renal plasma flow were found to accompany ACTH administration at 100 mg. dosage. In two studies striking increases of 65 per cent and 84 per cent, respectively, were found in the maximum reabsorptive capacity of the renal tubules for total ascorbic acid. This was in sharp contrast to reports in the literature of an increase in urinary ascorbic acid during administration of ACTH. The results will be discussed in terms of the effects of ACTH on the partition of plasma ascorbic acid into the reduced and oxidized forms and the significance of this in relation to the renal tubular enzyme systems involved in the reabsorption of ascorbic acid.

131. SYNDROMES INVOLVING HYPERNATREMIA

Harold M. Schoolman, M.D. (By Invitation), Alvin Dubin, M.S. (By Invitation), and William S. Hoffman, M.D.
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In recent years we have encountered 91 cases of unequivocal hypernatremia (hyperosmolarity) in acutely ill patients. Though the pathogenesis of this biochemical lesion was not entirely clear, it was obvious that a common

mechanism was not operative in all cases. There appeared to be at least three groups.

1. Primary desiccation from excessive loss of insensible perspiration without intake of fluids was seen in 12 infants and 10 adults. Most of the infants were younger than 6 months and were admitted for diarrhea. Loss of gastrointestinal fluid was apparently less important here than the lack of intake of fluid, for commonly in infantile diarrhea there is hyponatremia. In adults hypernatremia was seen during severely hot weather in patients ill under conditions precluding oral intake of fluids. Serum sodium ranged from 150 to 175 meq. per liter, chloride 118 to 133. When diarrhea was present chloride acidosis usually was seen, with serum bicarbonate levels below 10 meq. per liter.

2. Six patients in diabetic coma after treatment with insulin and saline solution alone showed sodium levels of 151 to 163, chloride 119 to 132 meq. per liter, quite as was found by Peter's group.

3. Thirty-five patients with marked hypernatremia showed evidence of an acute intracranial lesion. Some were initially diagnosed diabetic coma since the urine showed glucose and acetone. However, the serum bicarbonate was normal. It is possible that these patients had acute traumatic diabetes insipidus, though none lived long enough to allow extensive studies. An additional group of 28 patients showed initial hyperosmolarity but died before diagnosis could be established. Many probably had intracranial lesions.

The common findings in all groups were nitrogenous retention and listlessness or frank coma. Serum potassium showed no consistent pattern. The first two groups responded to injections of glucose solution, but the patients with intracranial lesions usually remained in coma even when the hypernatremia was relieved.

132. CLINICAL VALUE OF THE BUTANOL EXTRACTABLE (THYROXINE) I^{131} IN THE DIAGNOSIS OF HYPERTHYROIDISM

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(INTRODUCED BY E. B. FLINK, M.D.)

Thyroxine can be separated from non-thyroxine protein-bound iodine in plasma by extraction with butanol. In the present study the total plasma protein-bound radioactivity (PBI^{131}) and the butanol extractable fraction (BEI^{131}) were determined in 50 euthyroid and 11 hyperthyroid subjects twenty-four hours after the administration of an oral tracer dose of radioactive iodine. The basal metabolic rate and thyroid uptake of I^{131} at twenty-four hours were also determined in each subject for comparison.

For the euthyroid group the mean value of the BEI^{131} was 0.015 per cent per liter of plasma (range 0.001 to 0.034). The mean value of the PBI^{131} was 0.024 per cent per liter (range 0.005 to 0.060). For the hyperthyroid group the mean BEI^{131} was 0.251 per cent per liter (range 0.048 to 0.955) and the mean PBI^{131} was 0.368 per cent per liter (range 0.064 to 1.565).

In the hyperthyroid range the BEI^{131} correlated nearly perfectly with the PBI^{131} ($r = 0.997$) and was on the average two-thirds of the latter value. In the euthyroid range the average BEI^{131} was also approximately two-thirds of the PBI^{131}; however unlike the hyperthyroid group there was marked variability in the ratio of the two measures (range 0.20-1.00). The magnitude of the BEI^{131} to PBI^{131} ratio was thus unrelated to the state of thyroid function.

Both the BEI^{131} and PBI^{131} distinguished the hyperthyroid from the euthyroid patients more effectively than the twenty-four hour uptake of I^{131} and the BMR. However the BEI^{131} is no better than the PBI^{131} for this purpose and is technically more difficult.

133. THE ELECTROCARDIOGRAM IN MITRAL VALVE DISEASE

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(INTRODUCED BY JOHNSON MCGUIRE, M.D.)

Twenty-seven patients with mitral valve disease have undergone cardiac catheterization. The electrocardiographic patterns (in 12 lead tracings) encountered in these patients were compared with the resting pulmonary artery pressures, the pulmonary vascular resistances, and the mitral valve areas.

Fifteen patients had pure mitral stenosis. Seven of these showed the pattern of right ventricular hypertrophy and two showed incomplete right bundle branch block. Six of the seven with hypertrophy and one with incomplete right bundle branch block had total pulmonary vascular resistances in excess of 1,000 dynes second $cm.^{-5}$.

Nine patients had both mitral stenosis and insufficiency. One showed right ventricular hypertrophy, one showed both incomplete right bundle branch block and right ventricular hypertrophy, and two showed incomplete right bundle branch block. In two of these the total pulmonary vascular resistance exceeded 1,000 dynes seconds $cm.^{-5}$. Two other patients with total pulmonary vascular resistances exceeding 1,000 dynes seconds $cm.^{-5}$ showed neither right ventricular hypertrophy nor incomplete right bundle branch block.

Three patients had both mitral and aortic valve lesions. None showed right ventricular hypertrophy or incomplete right bundle branch block, yet two had total pulmonary vascular resistances well in excess of 1,000 dynes seconds $cm.^{-5}$.

Twenty-five of the cases studied had mean pulmonary artery pressures exceeding 30 mm. Hg, yet only thirteen developed the pattern of either right ventricular hypertrophy or incomplete right bundle branch block. Auricular fibrillation or abnormal P waves were encountered in all except four patients studied. Three of six patients with mitral valve areas of 0.5 $cm.^2$ or less showed the pattern of right ventricular hypertrophy.

In pure mitral stenosis if the total pulmonary vascular resistance exceeds 1,000 dynes seconds $cm.^{-5}$, the electrocardiogram will usually (although not invariably) show the pattern of either right ventricular hypertrophy or incomplete right bundle branch block. If mitral insufficiency and/or aortic valve lesions coexist with mitral stenosis, the total pulmonary vascular resistance may exceed the level of 1,000 dynes seconds $cm.^{-5}$ many times without the development of the pattern of right ventricular hypertrophy.

134. PHYSIOLOGIC MEASUREMENTS IN MITRAL VALVE DISEASE

RALPH C. SCOTT, M.D. (BY INVITATION), SAMUEL KAPLAN, M.D. (BY INVITATION), AND NOBLE O. FOWLER, M.D.
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Thirty-four patients with mitral valve disease were studied at rest (R) by cardiac catheterization. Nineteen had pure stenosis (I), 12 had stenosis and insufficiency (II), and three had combined aortic and mitral lesions (III).

Fourteen in I, seven in II, and all in III were studied after standard exercise (E).

Mean physiologic measurements for each group listed in sequence [(I R, E); (II R, E); (III R, E)] are as follows:

Cardiac index (liters/min./M.^{2}) = (2.5, 2.5); (3.1, 3.6); (1.7, 1.8).

Oxygen consumption (ml./min./M.^{2}) = (118, 169); (153, 223); (109, 167).

Arteriovenous oxygen difference (vol. per cent = (5.40, 6.70); (5.46, 6.78); (7.41, 9.77).

Mean pulmonary artery pressure (mm. Hg): (54, 58); (57, 75); (68, 73).

Mean right ventricular pressure (mm. Hg) = (36, 51); (36, 47); (48).

Mean right auricular pressure (mm. Hg) = (9, 13); (12, 14); (12, 13).

Pulmonary "capillary" pressure (mm. Hg) = (24, 44); (31, 43); (32, 33).

Pulmonary arteriolar resistance (dynes sec. cm.$^{-5}$): (747); (536, 316); (1290, 1259).

Total pulmonary resistance (dynes sec. cm.$^{-5}$) = (1357, 1456); (1135, 980); (2514, 2265).

Peripheral resistance (dynes sec. cm.$^{-5}$): (2080, 1759); (1661, 1098); (2681).

Right ventricular work (Kg. M./min./M.^{2}) = (1.6, 3.5); (2.0, 4.1); (1.2, 1.3).

Left ventricular work (Kg. M./min./M.^{2}) = (4.2, 5.7); (8.6); (10.6).

Forward mitral valve flow (ml./diastolic sec.) = (105, 140); (112); (167).

Mean regurgitant output of left ventricle (L./min./M.^{2}): (II 3.3); (III 1.9).

Twenty patients had mitral valvuloplasty. Comparison between mean mitral valve area (or forward area) estimated at surgery (S) and mean mitral valve area (or regurgitant area) as calculated (C) is as follows (cm.^{2}): (I (S) 0.5, (C) 0.7); (II 1.0, 0.6); (III 1.0, 0.8). Individual measurements often did not compare as closely as did the mean values.

Pulmonary vascular pressures and resistances were elevated in all patients; pressures rose further with exercise. Right ventricular work was normal or elevated in all and tended to increase with exercise. Average left ventricular work was normal in I, elevated in II and III. The cardiac index was low or normal in all and showed a fall or only slight increase with exercise. Total cardiac output (aortic plus regurgitant) in II and III was normal or increased.

135. INTRAVENOUS INFUSION OF COMPOUND F AS A MEANS OF DIFFERENTIATING HYPERADRENAL STATES

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A continuous infusion of approximately 50 mg. of compound F in 250 ml. of 1 per cent alcohol and 5 per cent dextrose was given over a 24-hour period to each of three female patients with hyperadrenocorticism. One patient had Cushing's syndrome due to a unilateral adrenocortical carcinoma; the other two had adrenal hyperplasia with virilism.

In each patient determinations of urinary 17-ketosteroids and pregnanediol chromogens (by the method of Somerville and Marrian) were made. Urines were collected as 6- or 12-hour specimens during the period of observation. The pretreatment 17-ketosteroid excretion, 13.4 mg. per 24 hours in the Cushing's, averaged 25 and 90 mg. per 24 hours in the virilized patients. Pre-

treatment pregnanediol chromogen averaged 1.65 mg. per 24 hours in the Cushing's syndrome, and 3 and 200 mg. per 24 hours in the hyperplasias respectively. The latter figures probably include pregnanetriol as well as pregnanediol.

The compound F infusion produced only slight changes in the steroid excretions in the Cushing's syndrome. In the hyperplasia cases, the 17-ketosteroid excretion dropped to 10.4 and 6.8 mg. per 24 hours, respectively, and pregnanediol chromogen decreased to 0 and 43.5 mg. per 24 hours, respectively. The lowest steroid excretion values were found in the 6- to 12-hour period immediately following the completed compound F infusion. The 17-ketosteroid values promptly returned to pretreatment levels within 24 hours after the infusion of compound F. The rise in pregnanediol chromogen excretion after compound F was much slower than the 17-ketosteroid rise.

In one hyperplasia patient, ACTH was given intramuscularly over a 48-hour period, after the pregnanediol chromogen had been previously suppressed by compound F. There was a marked rise in pregnanediol chromogen from 1.7 to 57.5 mg. per 24 hours.

The importance of these findings in the differential diagnosis of hyperadrenal states, and the possible theoretic implications in the genesis of adrenal hyperplasia will be discussed.

136. THE ELECTROCARDIOGRAM IN CONGENITAL PULMONIC STENOSIS

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(Introduced by Louis N. Katz, M.D.)

Electrocardiographic and cardiac catheterization data were compared in 40 cases of pulmonic stenosis, mostly associated with other congenital cardiac defects, in order to determine whether or not electrocardiographic patterns could be found pointing to specific lesions. Three such definite patterns, all characteristic of right heart strain, were found alone, or in combination, and could be correlated with the dynamic data. These patterns can be called (a) mild, (b) severe resistance overloading, and (c) input overloading. Our correlations suggest that characteristic electrocardiograms permit the prediction of pertinent dynamic findings in many cases of pulmonic stenosis. The diagnosis of severe pulmonic stenosis with completely intact septa can be made with remarkable certainty from the electrocardiographic pattern alone.

137. THE ENZYMATIC DISSOLUTION OF INTRAVASCULAR CLOTS

Sol Sherry, M.D., Antoinette Titchener, M.D. (By Invitation), Leonard Gottesman, M.D. (By Invitation), Philip Wasserman, M.D. (By Invitation), and Walter Troll, Ph.D. (By Invitation)
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Trypsin, chymotrypsin, and an activator of the naturally occurring plasma proteolytic enzyme (plasmin) were investigated in a large series of dogs for their ability to dissolve experimentally induced intravascular clots. These agents were intravenously administered into animals containing one-day-old femoral or popliteal artery thrombi, and observations made of their in vivo biochemical action, as well as the gross and histological effects on the local thrombosis.

Trypsin slowly infused on two successive days in doses (16-33 mg./kg.) sufficient to produce a significant increase in circulating proteolytic activity, rendered the blood incoagulable (fibrinogen and prothrombin destroyed) but was entirely incapable of effecting clot lysis or producing significant changes in the natural course of the thrombus. Very small doses (0.4 mg./kg.), reported to be useful clinically, were not accompanied by increased circulating proteolytic activity, and had no effect on the thrombus.

Chymotrypsin infused in a similar manner, in amounts (12-30 mg./kg.) sufficient to establish circulating levels of proteolytic activity of the order obtained with the larger doses of trypsin, did not render the blood incoagulable (prothrombin intact, fibrinogen reduced 30 to 50 per cent) but produced a partial to complete lysis of the clot in approximately half the animals studied. Smaller doses of chymotrypsin (3-8 mg./kg.) did not significantly alter the thrombi.

The most striking effects were observed in the group in which immediate activation of plasmin was accomplished by an injection of a mixture of streptokinase and a partially purified human plasma Fraction III factor. Although either substance alone, in large doses, had no effect as an activator for the dogs' proteolytic enzyme system, their combination, in relatively small doses, resulted in a potent activator. Following a single rapid injection of this activator, increased circulating proteolytic and fibrinolytic activity were demonstrable for one to two hours. Circulating fibrinogen rapidly disappeared, and was accompanied by a transient incoagulable state of four to six hours' duration. Extensive lytic effects on the experimental thrombi were noted in the majority of the animals, with half of the clots completely lysed. No significant toxic or hemorrhagic manifestations were observed.

138. STUDIES ON THE USE OF ETHAVERINE IN ANGINA PECTORIS

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Ethaverine, the ethyl analogue of papaverine, was submitted to clinical evaluation in 22 patients from the Cardiac Clinic of the Cincinnati General Hospital with well-established angina pectoris.

The patients were observed at weekly intervals and kept daily records of the number of nitroglycerin tablets used, on a calendar-type card. Doses ranged from 200 to 400 mg. daily in divided dosage.

Following a control period of approximately six weeks either ethaverine or a placebo was administered to each patient for a similar period, following which the patient was given the other material for an additional six weeks. Neither the patients nor the physician knew which was being given and the findings were not evaluated until the study was complete. Modified Master tests were carried out on each patient during each of the three periods of the study. Results: All of the patients showed a significant improvement over their control periods during the administration of either ethaverine or placebo. However, no statistically significant difference was found between the number of tablets used during the period the patients were on the drug and on the placebo. The Master tests, which were positive in most cases, were uninfluenced by medication. There were no significant side effects observed.

139. THE EFFECT OF CARBON DIOXIDE UPON HEPATIC BLOOD FLOW

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Previous observations have indicated that increase of carbon dioxide blood levels during surgical anesthesia increases the frequency and severity of hepatic injury appearing postoperatively. Administration of high concentrations of carbon dioxide alone did not produce demonstrable liver injury. Measurements of hepatic blood flow have been made utilizing the bromsulfalein clearance method of Bradley et al. Observations were made during inhalation of atmospheric air, during the administration of carbon dioxide in concentrations of 7 and 33 per cent, with oxygen, during anesthesia while blood carbon dioxide content was normal, and during administration of anesthesia plus a high concentration of carbon dioxide. In no subject were more than two of these situations evaluated. The rate of hepatic blood flow varied widely from subject to subject, but in every instance the existing rate of flow in a given subject was significantly decreased when carbon dioxide in abnormally high concentrations was added to the atmosphere being inhaled. Additionally, the observation of others that surgical anesthesia alone decreases the rate of hepatic blood flow was confirmed.

140. SOME OBSERVATIONS ON MALNOURISHED PATIENTS WITH EDEMA

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Eight famine victims in Calcutta were selected on the basis of (1) malnutrition resulting from inadequate diet and (2) the development of edema in the course of the illness. Their diet was basically rice supplemented with pulses. Protein and caloric deficiencies were striking.

Clinical cardiac findings were normal, including venous pressures and circulation times. Electrocardiographic and neurologic changes were minor and variable. The urine and liver function studies were normal. There was the expected reduction in serum proteins and plasma volume accompanied by an increased thiocyanate space. These changes were followed during recovery brought about by dietary control, and related to body weight and fluid intake and output. In some patients the oliguria present on admission continued for about two weeks. In others, diuresis occurred at onset of treatment, accompanied by a reduction in weight and in the thiocyanate space but by little change in the plasma volume. In four such patients the weight stabilized and remained constant or increased only slightly for some days, as long as two weeks, during which time the minimal edema gradually receded, the thiocyanate space diminished, and plasma volume increased. In these periods diuresis was constant and disproportionately great for the change in thiocyanate space. Likewise in two patients whose clinical edema had entirely cleared, diuresis continued during a period of relatively constant weight, and plasma volume and thiocyanate space did not change appreciably. In two patients two waves of recurring clinical edema followed a primary diuresis during which the clinical edema present on admission had cleared entirely. These were accompanied by no appreciable change in body weight, and presumably resulted from internal shifts in body water.

These data indicate that in the edema studied, important water shifts occurred other than those between vascular and extracellular space, pre-

sumably between tissue cells and the extracellular space. Constant water loss occurred during a considerable phase of recovery, again presumably from the tissues. The data suggest the importance of the participation of the tissue cells in the edematous state studied and that in the fundamental mechanism of the edema this participation was important.

141. CONTROL OF GASTRIC ACIDITY BY A NEW WAY OF ANTACID ADMINISTRATION

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The therapeutic regimes in peptic ulcer patients aim at decreasing or neutralizing free acidity, since high acidity is considered not only as a factor in ulcer formation but also a hindrance in its healing. The high rate of relapse of peptic (duodenal) ulcer, is believed to be due to poor control of free acid in the stomach.

The present therapeutic regimes are inadequate for continuous control of gastric acidity. Even the hourly ingestion of an antacid frequently fails to keep the gastric juice completely neutralized. In some patients the acidity is neutralized for only fifteen to thirty minutes following the ingestion of an antacid or of several ounces of milk.

The most effective method of neutralizing the stomach contents over long periods of time has been the continuous intragastric milk and alkali drip introduced by Winkelstein. This method, however, while effective and well tolerated has obvious disadvantages for many patients.

The recent introduction of a tablet, made up of milk solids and alkali, which is to be kept between the gums and the cheek and left to dissolve on its own represents the nearest approach to the continuous intragastric drip for the ambulatory patient.

We are now studying the changes in gastric acidity and clinical results in a small number of patients receiving these tablets which contain solids from whole milk, combined with dextrins and maltose, magnesium trisilicate, magnesium oxide, calcium carbonate, magnesium carbonate, and peppermint oil. Most patients tolerate them well and are cooperative in taking from 10 to 16 tablets per day. The tablets last twenty-five to thirty-five minutes before they are completely used up. To date the acid curves show a drop of the acidity within 15 minutes and thereafter an almost complete neutralization of the gastric juice in the histamine stimulated stomach, during the time the tablets are dissolving in marked contrast to the brief control exerted by other antacid tablets.

This method of taking antacids may prove a very useful one for some ulcer patients.

142. THE EFFECT OF ERYTHROMYCIN IN AMEBIASIS

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The therapeutic effects of erythromycin in amebiasis were studied in a total of 30 patients—eight with acute and 22 with chronic infection.

Three stools were taken prior to beginning of the treatment, and monthly intervals thereafter for three months. Five hundred milligrams of erythromycin were given every six hours for a period of five days. In some patients a course of diodoquin (90 grains daily for 10 days) followed the course of erythromycin.

Prompt symptomatic response to erythromycin occurred in seven of the acute and in 20 of the chronic cases. Three patients did not return for follow up. Of the 27 patients whom we were able to follow for three months or longer, all were negative after the first and second month. Two of the relapsed cases belonged to the acute and five to the chronic group. One patient of the chronic group had negative stools six months after this continuation of the treatment. Of particular interest was the rapid disappearance of the ulcerative lesions, as seen proctoscopically, in the acute cases.

Erythromycin caused only minimal side effects. Some patients had flatulence and softening of the stools during the period of medication but these symptoms disappeared promptly after the cessation of therapy and there were no late sequelae.

From the small number of cases observed to date, this drug appears to be a useful substance for the treatment of amebiasis, especially in those cases in which an ulcerative colitis is associated.

143. STUDIES OF QUINIDINE PLASMA LEVELS AND RATE OF DECLINE FOLLOWING CESSATION OF ADMINISTRATION

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Since quinidine is most often used in patients with cardiac disease, it is of importance to know whether such individuals excrete or destroy quinidine in an abnormal manner. Accordingly, oral quinidine sulfate was administered in a fixed dosage schedule for 48 hours to four groups of individuals: (1) healthy control group, (2) group of cardiac patients, (3) group of patients with obvious renal failure, and (4) a group of patients with frank liver disease. Plasma quinidine levels were determined two hours, twenty-six hours, and fifty hours after the last dose of quinidine. The experiment was planned to determine the peak plasma level reached by each group on this dosage schedule, and to analyze the rate at which the body in equilibrium with a known quantity of quinidine removed the drug from the blood. In addition it was noted whether cardiac, hepatic or renal failure altered either of the first two determinations.

Each of the four groups had approximately the same mean peak quinidine level of 4.1 mg. per liter. The rate of decline in the plasma level was less rapid in the cardiac patients than in either normal persons or patients with liver or kidney disease. It does not seem likely then that either hepatic or renal changes secondary to heart failure are the factors responsible for the slower loss of the drug from the blood of cardiac patients. This slower declining level of quinidine in the blood of cardiac patients is not a factor of slower excretion and does not result in a more pronounced cumulative effect in such patients.

144. AN EVALUATION OF THE RESTORATION OF SINUS RHYTHM IN PATIENTS WITH AURICULAR FIBRILLATION BY THE USE OF QUINIDINE

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Quinidine sulfate was administered orally to a group of patients with chronic auricular fibrillation in an attempt to convert the cardiac rhythm to sinus rhythm. Congestive failure was treated and conversion was not attempted until the patient's clinical condition, weight, vital capacity, heart rate, and blood pressure revealed no improvement on three successive days.

Thirty-five cases with auricular fibrillation were studied. Four patients converted to sinus rhythm spontaneously while eighteen patients converted to sinus rhythm during quinidine therapy. The majority of conversions occurred at levels below four milligrams per cent. In those patients in whom sinus rhythm was restored, cardiac enlargement was not detectable in ten, moderate enlargement was present in ten and gross enlargement (apex at the anterior axillary line) was detectable in two patients. Thirteen patients failed to convert to sinus rhythm with effective quinidine blood levels. Two of these had moderate enlargement and eleven had great enlargement of the heart. The incidence of failure to convert to sinus rhythm was much higher in patients with a grossly enlarged heart.

There was no noticeable improvement in the group of patients who were converted to sinus rhythm either subjectively or as shown by study of the objective criteria used.

A comparative study of the electrocardiograms of these patients was made before, during and after this quinidine therapy. A correlation between plasma level and changes observed in the constituent portions of the electrocardiogram was attempted. A high incidence of conduction defects, especially of auricular origin, was observed in those cases successfully converted to sinus rhythm. Irrespective of the rhythm there was a marked correlation between ST-T segment changes and the plasma quinidine level. The value of the electrocardiogram in indicating probability of conversion of a given case of auricular fibrillation was low.

145. INCREASED CEREBROSPINAL FLUID PRESSURE AND PAPILLEDEMA AS INDEPENDENT MANIFESTATIONS OF MALIGNANT HYPERTENSION

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The papilledema of the malignant phase of hypertensive disease has been attributed to increased cerebrospinal fluid pressure which Pickering finds has "a close correlation" with diastolic arterial pressure. The available data do not prove this contention.

Consequently, cerebrospinal fluid pressure (CSFP) was measured in 100 hypertensive patients with relatively benign disease and in 100 in the malignant phase with papilledema. Repeated measurements were made in 20; 90 were made in one. The average of diastolic morning and evening pressures on the day of observation was then associated with CSFP.

Measurements were also made in one patient during the onset of papilledema and in one during its advance from 2 to 4 plus severity; observations were made in 5 during recession of papilledema brought about by pyrogen and in 2 of these during its recurrence.

Normal maximum CSFP was taken as 250 mm. of water; CSFP of 87 of the patients without papilledema was normal as it was also in 57 of the patients with papilledema. Thus, there is no constant association between elevated CSFP and the occurrence of papilledema.

The correlation between CSFP and mean diastolic pressure was then examined. No association is demonstrable in patients without papilledema. In those with papilledema, the correlation coefficient (r) is 0.347; the correlation is significant since r/ standard error of r is 3.5. This association does not establish a causal relationship; thus, mean diastolic pressures of 160 mm. Hg are sometimes associated with CSFP less than 250 mm. of water and diastolic pressures of 110 mm. Hg with CSFP in excess of 300 mm. of water. Further, neither the onset nor the recession of papilledema could be associated with diastolic pressure in serial observations. Variations of CSFP from 50 to 450 mm. of water were observed in 1 patient during periods in which diastolic pressure was relatively constant.

In conclusion, increased diastolic pressure as such is not a cause of increased CSFP; increased CSFP is not a cause of papilledema in patients with the syndrome of malignant hypertension. The association between these phenomena suggests that both are common but independent sequelae of the vascular damage incident on malignant hypertension.

146. JAUNDICE COMPLICATING ALCOHOLIC STEATOSIS OF THE LIVER

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Jaundice occurring in the presence of fatty infiltration (steatosis) of the liver associated with chronic alcoholism produces a syndrome which has received scant attention. It is a syndrome of importance in the differential diagnosis of obstructive jaundice which it may mimic closely, and it calls for urgent treatment owing to the tendency of these patients to pass rapidly and unexpectedly into coma and die.

In a study of 23 cases proved by needle biopsy of the liver or autopsy and 11 cases judged to be identical on clinical grounds, the hepatic failure was nearly always precipitated by some infection, usually upper respiratory, and it appeared that the liver had been rendered especially vulnerable by a preceding period of acute-upon-chronic alcoholism associated with almost complete lack of food intake.

Clinically, the outstanding features were evidence of recent infection, marked icterus, gross hepatomegaly, often exquisitely tender, without significant splenomegaly or ascites, and severe bilirubinuria often without urobilinogenuria. Spider nevi and fetor hepaticus were infrequent. The hepatic tests were often similar to those in obstructive jaundice with one-third of the cases showing negative cephalin flocculation, thymol turbidity below 5.0 units and alkaline phosphatase above 10.0 Bodansky units. The bilirubin level rose up to 30.0 mg. per 100 ml. serum and jaundice per-

sisted for many weeks in the surviving patients. The serum albumin was above 3.5 Gm. per 100 ml. in 38 per cent of the cases, the serum globulin was less than 3.4 Gm. in 56 per cent, and the albumin-globulin ratio was not reversed in 41 per cent. The gamma globulin was usually elevated but seldom above 2.0 Gm. per 100 ml. serum.

The prognosis in the more severe cases appeared to depend mainly upon the response of the infection to antibiotic therapy. A short course of intravenous aureomycin was effective and no hepatotoxic effects from this therapy were observed.

147. THE EFFECT OF VITAMIN B_{12}, FOLIC ACID, AND ASCORBIC ACID ON THE NUCLEIC ACID COMPOSITION OF THE BONE MARROW OF PATIENTS WITH MEGALOBLASTIC ANEMIAS

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There is considerable evidence indicating that folic acid derivatives are important catalysts for the formation of the purine ring and that they are involved in pyrimidine metabolism also. Vitamin B_{12} likewise is involved in pyrimidine metabolism. Ascorbic acid has its impact on nucleic acids presumably through its recognized role in folic acid metabolism. For these reasons, we have investigated the effect of these nutrients on the nucleic acids and pyrimidines of bone marrow obtained from patients with megaloblastic anemia in relapse.

Ten patients with megaloblastic anemia have been studied. RNA and DNA were measured in the buffy coats of bone marrow aspirates by the orcinol and diphenylamine reactions. Uracil and thymine (and other pyrimidines and purines) were determined by a paper chromatographic method modified to meet the requirements of bone marrow cell analyses. Molar ratios of RNA:DNA and U:T were calculated and will be correlated with the morphologic appearance of the marrow. These determinations were made one or more times during therapy with the hematopoietic vitamins given in both optimal and suboptimal amounts. The bone marrow from 6 normal persons served as controls. The following results have been obtained: (1) U/T and RNA/DNA ratios of normal marrow specimens range from 0.18 to 0.05 and 0.47 to 0.23, respectively. (2) In patients with megaloblastic anemia in relapse these values are 0.41 to 0.26 and 1.0 to 0.61, respectively. The more severe the relapse, the higher are the ratios. (3) Effective therapy with vitamin B_{12} or folic acid reduces these ratios to the normal range before the reticulocytic response occurs. Suboptimal therapy induces gradual reduction. The total nucleic acids after therapy have a lower concentration of uracil and a greater concentration of thymine than before therapy.

These results provide chemical proof of the importance of Vitamin B_{12} and folic acid to nucleic acid metabolism in human beings. Since the U/T ratio differs from the RNA/DNA ratio in both normal and P.A. patients, additional data are added to the rapidly accumulating evidence that the tetranucleotide theory of nucleic acid structure is incorrect.

148. A CHROMOSOME MAP OF MICROCOCCUS PYOGENES BASED ON DETERMINATIONS OF THE SENSITIVITIES OF INDIVIDUAL STRAINS TO ANTIBIOTICS

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(INTRODUCED BY FRANCIS D. MURPHY, M.D.)

The chromosome map to be presented is based on the following assumptions: (1) the sensitivity or resistance to antibiotics of bacteria is under gene control (Demerec: J. Bact. **56**: 163, 1948); (2) the arrangement of genes in bacteria is in linear order on a single chromosome (Lederberg: Genetics **32**: 505, 1947); (3) samples of the population of *M. pyogenes* drawn from infections in human beings represent individual clones of a population that is undergoing a relatively rapid evolutionary change. The characteristics of these clones are determined by sexual reproduction (Lederberg: Genetics **32**: 505, 1947); (4) a comparison of the numbers of times resistances and sensitivities to various antibiotics are associated in the various strains will reveal the position on the chromosome of the theoretic "wild type" of the genes controlling reactions to these antibiotics.

The data were obtained from the results of in vitro tests of the sensitivity to antibiotics of strains of *M. pyogenes* isolated from patients on the wards of the Milwaukee County General Hospital. Originally the results were used to help the ward physician decide which of the antibiotics was most likely to eradicate the infections from which the strains were isolated. The data were then considered from the viewpoint of a chromosome map and were found to fit a map that placed the loci of the reactions to antibiotics in the following order: chloramphenicol resistance, chloramphenicol sensitivity, neomycin sensitivity, penicillin resistance, streoptomycin sensitivity, streptomycin resistance, oxytetracycline resistance, aureomycin resistance, oxytetracycline sensitivity, penicillin sensitivity, aureomycin sensitivity, and neomycin resistance. When the data were arranged in a table to reveal the crossover percentages, a method of determining the order in which antibiotics should be administered to patients with severe infections due to *M. pyogenes* became apparent and will be presented.

149. A PRELIMINARY STUDY ON THE RENAL EXCRETION OF RADIOACTIVE COLCHICINE IN PATIENTS WITH AND WITHOUT NEOPLASTIC DISEASE

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CHICAGO, ILL.

It was previously reported by one of us (E. J. W.) that the metabolism of radioactive colchicine in normal mice differed markedly from that found in tumor-bearing mice. With these basic findings, it was deemed advisable to study the renal excretion of radioactive colchicine in selected patients with and without neoplastic disease. In this preliminary report, we wish to present data on the excretion of radioactive colchicine in four patients. In this group were two patients with extensive neoplastic lesions. The other two had bronchial asthma but were free of neoplastic disease. The radioactive colchicine was injected intravenously in a dose of 3 mg. per patient. Individual urine samples were collected for 48 hours. They were then made

up to suitable volumes and aliquots taken for analysis. One aliquot was combusted and total C^{14} determined, the other was extracted for its colchicine content.

It was found that the two tumor patients excreted 4.8 per cent and 3.88 per cent of the administered dose as unchanged colchicine. The two normal patients excreted 46.77 per cent and 15.6 per cent of the administered dose as unchanged colchicine. Thus far, these data compare favorably with those found in animals which showed a sixfold higher excretion for normal mice over those in tumor-bearing mice. No C^{14} was found in the expired air and in most instances, 97 per cent of the C^{14} can be accounted for in the 48 hour urine. No detectable quantity of C^{14} was present in urea isolated from the patients' urine.

Data on additional patients will be presented at the meeting.

150. SYSTEMIC EFFECTS FROM HYDROCORTISONE ACETATE ADMINISTERED INTRA-ARTICULARLY TO RHEUMATOID PATIENTS

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Our experience suggests that a portion of hydrocortisone acetate injected intra-articularly is absorbed from the synovial cavity and produces systemic effects.

Generalized antirheumatic effects were noted in 21 per cent of 148 rheumatoid patients who received hydrocortisone acetate intra-articularly. These effects, usually mild, were similar in nature to those produced by cortisone or hydrocortisone acetate administered orally or intramuscularly. The most common general manifestation was diminution of stiffness, tenderness and pain even in those rheumatoid joints which had not been the sites of injection; range of motion frequently improved and synovial swelling occasionally lessened in those joints. Fever was reduced on several occasions in 1 patient. Effects were noted within two to twenty hours after injection, and usually persisted about one day and sometimes two days.

Notable factors influencing the occurrence and degree of systemic effects were the following:

1. Dosage. Ninety per cent of systemic reactions were noted after injection into one or more joints of total doses of 50 mg. or more of the agent, whereas only 10 per cent were noted after total doses of 37.5 mg. or less had been given, although the latter doses were used in 35 per cent of injections. Likewise, most marked effects usually followed larger doses.

2. Susceptibility to effects of the hormone. Certain rheumatoid patients seemed to be more susceptible to the effects of these hormones given parenterally, for example, women in general and patients with hypercortisonism who were undergoing gradual reduction in the dose of the hormone. These same groups were more likely to note systemic effects after intra-articular injections. The degree of local antirheumatic effect did not influence consistently the incidence or degree of systemic effect.

The urinary excretion of corticosteroids and 17-ketosteroids was studied in 3 patients who received intra-articular injections of 75, 150, and 150 mg. of hydrocortisone acetate, respectively. The excretion of corticosteroid during twenty-four hours after injection was increased significantly in all 3 cases, being 2.0, 1.19, and 1.43 mg., respectively, compared to control levels of 0.68, 0.74, and 0.94 mg., respectively. Injection (175 mg.) was

repeated in the third case; the excretion of corticosteroid increased from 0.78 mg. (preinjection) to 2.58 mg. (postinjection). The excretion of 17-ketosteroids was not consistently altered in these cases.

151. PNEUMONIA IN NAVY RECRUITS: ETIOLOGY AND CONTROLLED EVALUATION OF THERAPY

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GREAT LAKES, ILL.

(INTRODUCED BY CLAYTON G. LOOSLI, M.D.)

Seventy-seven consecutive cases of pneumonia were observed in Navy recruits between Dec. 13, 1950, and Feb. 26, 1951, and Nov. 7, 1951, and April 22, 1952. During the first year, 23 patients were treated with aureomycin or placebos and during the second year 54 patients were treated with aureomycin or penicillin in strict alternation.

Laboratory procedures in each case included culture of sputum; intraperitoneal inoculation of sputum into white mice; inoculation of nasal washings into embryonated eggs; and determination of cold agglutinin, antistreptolysin 0, influenza antihemagglutinin, and heterophile antibody titers.

Beta hemolytic streptococci were isolated from the sputum in 15 cases during the second year of the study, but this occurred when the carrier rate was high and clinically they were not typical of streptococcal pneumonia. Pneumococci were isolated infrequently and in only 4 instances were low numbered types isolated. Influenza as demonstrated by virus isolation or serology occurred in 3 cases. Cold agglutinins in significant titer were present in only 4 cases. The white blood count was above 12,000 in 60 per cent of the cases. Roentgenographic lesions were generally in the lower lobes and in multiple lobes in only 16 per cent of the cases. The roentgenographic interpretation was generally nonbacterial pneumonia.

During the first year of study, the response to aureomycin therapy appeared dramatic when compared to the placebo-treated control group. During the second year, aureomycin and penicillin were equally effective in promoting a rapid clinical recovery.

Although the study was initiated for controlled evaluation of aureomycin therapy in patients with primary atypical pneumonia, the results indicated that the incidence of primary atypical or viral pneumonia was negligible and that response was excellent with either aureomycin or penicillin therapy. It was difficult in the majority of cases to establish an etiological diagnosis despite extensive laboratory procedures. The results indicated that these cases could not simply be dismissed as viral in origin. Pathogenic bacteria were isolated from some of the patients, but generally it was not possible initially to identify these cases on the basis of clinical or laboratory findings.

152. RED CELL SURVIVAL STUDIES IN ABNORMAL HEMOGLOBIN SYNDROMES

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(INTRODUCED BY LEON O. JACOBSON, M.D.)

The red blood cell survival time in patients with sickle-cell anemia, sickle-cell trait, sickle cell-hemoglobin C disease, and hemoglobin C trait has been investigated with the use of the $NaCr^{51}O4$ tracer technique.

The abnormal hemoglobins present in these patients were determined by the alkaline denaturation and filter paper electrophoresis methods.

Red cell survival time in these patients correlated well with the type of abnormal hemoglobin present and also with severity of the hemolytic process as judged by the usual clinical and laboratory methods.

These results add additional information to the rapidly increasing knowledge concerning the newer abnormal hemoglobin syndromes.

The relatively new $NaCr^{51}O4$ tracer technique for studying red cell survival will be demonstrated and its applicability to this type of investigation stressed.

153. THE OCCURRENCE OF APPARENT CHRONIC MYELOID LEUKEMIA IN MICE FOLLOWING INTERMITTENT CORTISONE THERAPY

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In mice cortisone produces granulocytosis, lymphopenia, and myeloid metaplasia in the liver and spleen. When therapy is discontinued the normal hematologic picture is restored. With this knowledge the hypothesis was made that cortisone might produce permanent changes if given intermittently over a long period of time. In two preliminary experiments this seemed to be the case. After about 7 months of intermittent cortisone therapy permanent granulocytosis was established in a few animals which persisted for several months after cortisone was discontinued. However, most of the animals died before this change was established, probably due to the high dose of cortisone, 1.0 mg. daily.

In a third experiment 15 weanling mice of a nonleukemic Swiss strain were treated intermittently with cortisone, 0.5 mg. daily (H) for 10 months. There were 14 treatment periods averaging 7.3 days with rest periods in between averaging 16 days. Eleven of the mice survived 8 months or more; one survived 15½ months. In the early months the usual granulocytosis and lymphopenia occurred with therapy and reverted to normal in the rest periods. After 7 months the granulocytosis became permanent and persisted until 15½ months when the last mouse died, even though cortisone had been discontinued at 10 months. Lymphopenia did not persist after cortisone was discontinued. In two of the mice which survived 12½ and 13 months the liver and spleen showed histologic findings which were practically indistinguishable from those of human chronic myeloid leukemia. In a third mouse which survived 12 months similar infiltration of the liver occurred to a slight degree. In mice from the same strain and of ages comparable to the experimental animals similar abnormal hematologic and histologic findings did not exist.

154. UTILIZATION OF OPTICAL ISOMERS OF CYSTEINE BY LEUKOCYTES

AUSTIN S. WEISBERGER, M.D., AND LEIF G. SUHRLAND, M.D. (BY INVITATION)
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L-cysteine modifies the leukopenia induced by nitrogen mustard (HN2). Of the compounds studied, this protective effect occurs only in those containing closely opposed sulfhydryl, amino, and carboxyl groups. Alteration or substitution of any of these radicals results in complete loss of protection. This protective effect may be due to chemical inactivation of HN2 or it may be due to a more specific mechanism such as competitive inhibition of HN2. Therefore, the structural specificity required for this protective effect was further investigated by comparing the effect of D-cysteine with that of L-cysteine against HN2-induced leukopenia.

Equimolar amounts of D-cysteine were not as effective as L-cysteine in modifying leukopenia and neutropenia in rabbits. The average fall in the leukocyte count when HN2 was given without cysteine was 79.7 per cent of the initial value. When L-cysteine was injected prior to HN2 the average fall in the leukocyte count was 35.9 per cent compared with a fall of 61.6 per cent when D-cysteine was injected. The average fall in neutrophils was 79.8 per cent of the initial value with HN2. With L-cysteine the fall in neutrophil count was 37.3 per cent and with D-cysteine 82.8 per cent. It therefore appears unlikely that L-cysteine protection is due solely to chemical inactivation of HN2.

The failure of D-cysteine to exert as much protection as L-cysteine may be due to nonutilization of the dextrorotatory isomer by leukocytes. This was investigated by studying the effect of nonisotopic cysteine on the uptake of S^{35} labeled L-cystine by leukemic leukocytes in vitro. Prior incubation and saturation of leukocytes with nonisotopic L-cysteine reduces the uptake of S^{35} L-cystine. Prior incubation with equal amounts of D-cysteine does not result in a reduced uptake of S^{35}.

The data indicate that a specific spatial configuration as well as certain structural components is required to prevent the leukotoxic effect of HN2. The utilization of L-cysteine by leukocytes and the nonutilization of D-cysteine are further evidence that L-cysteine may have a unique role in the metabolism of leukocytes.

155. STUDIES ON THE UTILIZATION OF LABELED L-CYSTINE BY LEUKEMIC LEUKOCYTES IN VITRO

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Previous studies indicate that L-cysteine or related compounds may have an important role in the metabolism of leukocytes. Thus L-cysteine modifies the leukopenia induced by nitrogen mustard and this effect depends upon the presence of certain spatial and structural components. In vivo studies with S^{35} labeled L-cystine demonstrate a very rapid uptake of S^{35} by leukemic leukocytes compared with that of normal leukocytes. Because of these findings the in vitro utilization of S^{35} labeled L-cystine by leukemic leukocytes was studied.

Labeled L-cystine was added to heparinized leukemic blood. Following incubation the leukocytes were separated and the radioactivity per milliliter packed leukocytes was determined. The influences of temperature, time of incubation, amount of S^{35} L-cystine added, pH, electrolyte concentration, and oxygen tension were studied.

Optimum uptake of S^{35} occurred at 37° C. Incubation with increasing amounts of S^{35} L-cystine resulted in an increased uptake by leukocytes. Saturation occurred when approximately 0.02 micromole of S^{35} L-cystine was added per milliliter blood. Increase in time of incubation resulted in an increased uptake with equilibrium being attained in thirty minutes. The uptake was depressed in the presence of low oxygen tension and increased with high oxygen tension. Variations in pH, electrolyte and glucose concentration within a physiologic range had little effect.

Prior incubation of leukocytes with nonisotopic L-cysteine diminished the uptake of S^{35} L-cystine. Prior incubation with D-cysteine or L-methionine had no effect. Leukocytes readily utilized labeled L-methionine but did not utilize appreciable amounts of S^{35} inorganic sulfate.

The in vitro utilization of labeled L-cystine appears to be an active process and not solely dependent upon diffusion. These observations are further evidence that L-cystine or related compounds are involved in leukocyte metabolism. Since reproducible results are obtained under constant conditions, compounds capable of interfering with the utilization of labeled L-cystine may be studied by this method.

156. STUDIES OF THE FUNCTION OF THE SPLEEN IN THE HEMOLYSIS OF RED CELLS IN HEREDITARY SPHEROCYTOSIS AND SICKLE CELL DISORDERS

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Erythrocytes freshly obtained from the pulp of surgically removed spleens were studied in an investigation of the role of the spleen in the destruction of erythrocytes of patients with hereditary spherocytosis, sickle cell trait, and sickle cell anemia.

From 5 to 7 per cent of red cells obtained from the splenic pulp of three idiopathic thrombocytopenia (ITP) patients, studied as controls, showed slight increases in osmotic fragility and spheroidicity. A fourth ITP patient was transfused forty-five hours before splenectomy with 250 ml. of red cells, identifiable by differential agglutination, from a splenectomized donor with hereditary spherocytosis. At the time of operation the peripheral blood contained 3 per cent donor cells compared to 49 per cent in the splenic pulp. The sequestered donor cells obtained from the spleen showed marked increases in osmotic fragility and spheroidicity compared to their pretransfusion characteristics.

A patient with esophageal carcinoma was transfused eighteen hours before elective splenectomy with 200 ml. of packed erythrocytes from a donor with sickle cell *anemia*. At operation peripheral blood contained 3 per cent and splenic pulp 8 per cent of sickle cells. Erythrocytes from the splenic pulp showed some increase in osmotic fragility and spheroidicity. By contrast, there was no splenic sequestration nor increase in osmotic

fragility of erythrocytes from the splenic pulp of a fifth ITP patient transfused eighteen hours before splenectomy with 500 ml. of blood from a donor with sickle cell *trait*.

The peripheral blood of a patient with sickle cell *anemia*, transfused with 2,000 ml. of normal whole blood before splenectomy, contained 43 per cent sickle cells with a decreased osmotic fragility. In contrast, splenic-pulp samples contained 60 per cent sickle cells which showed an extreme increase in osmotic fragility with hemolysis beginning in isotonic saline.

Erythrocytes of sickle cell *trait* which require low oxygen tensions for sickling were not sequestered nor altered osmotically within the spleen. Sickle-cell-*anemia* erythrocytes, which sickle and become viscous at physiologic oxygen tensions, and hereditary spherocytosis cells with increased thickness, are sequestered in the spleen, probably because of these physical characteristics. The increased susceptibility of sequestered cells to osmotic hemolysis presumably results from stasis within the spleen.

157. INCIDENCE OF HYPERTENSION AND DIABETES IN NEGROES WITH ACUTE MYOCARDIAL INFARCTION

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A study was made of the frequency of antecedent hypertension and/or diabetes in acute myocardial infarction in Negro men and women. In 90 per cent of each sex, hypertension and/or diabetes preceded the attack. The incidence of these factors in Negro men is similar to their incidence in white women who have had an acute myocardial infarction. We cannot offer an immediate explanation for the similarity.

158. THE INFLUENCE OF DIET ON THE BLOOD PRESSURE AND HEART RATE OF NORMAL DOGS. I. PROTEIN AND CARBOHYDRATE

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Omaha, Neb.

Dogs were fasted until the blood pressure decreased to the stable fasting level before the experimental diets were given. This has three advantages: (1) a more constant control level of blood pressure; (2) the momentum of the "metabolic mill," which may conceal specific food effects, is diminished; (3) fasting is an alarming stimulus which sensitizes the animal, so that specific, potential, effects of the food materials are intensified. The diets consisted of fresh horse meat, cracker meal, or rice and were fed at two levels of caloric intake; (a) low maintenance level (60 cal/M^2/hr./24 hr.) which stopped weight loss but allowed no gain; (b) luxus consumption level (twice the low maintenance) which allowed a progressive gain in weight. The protein and carbohydrate diets were approximately isocaloric at both levels. When the low maintenance diets were fed, blood pressure and pulse rate rose moderately above the fasting level but there was no consistent difference between protein and carbohydrate. When the luxus

consumption diets were fed, the blood pressure and pulse rates were significantly higher on the carbohydrate than on the protein. Systolic pressure was raised more than diastolic. Rice or cracker meal were equally effective and the high blood pressure could be maintained for over one hundred days without decreasing. Body weight of the animals was practically the same on both diets. If the blood pressure was elevated by giving the high carbohydrate diet after fasting, substitution of a high protein diet resulted in lowering of the blood pressure to levels characteristic of the protein diet. If the high carbohydrate diet was given without the preliminary fast, the elevation of blood pressure and pulse rate was transient, lasting only twelve to sixteen days. It is suggested that activation of the pituitary-adrenal system by the preliminary fast is necessary for the maintenance of the high level of blood pressure by carbohydrate and for the observed difference between the effect of carbohydrate and protein.

159. THE ALTERATION OF ASCORBIC ACID OXIDATION IN PERNICIOUS ANEMIA

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CINCINNATI, OHIO

The biochemical relationship of ascorbic acid and its oxidation product, dehydroascorbic acid, to hematopoiesis has not been established. Ascorbic acid seems to be necessary for the conversion of folic to folinic acid; a deficiency of vitamin C plays an important role in the etiology of the megaloblastic anemia of infancy, and minor hematologic responses may occur in pernicious anemia patients fed large doses of ascorbic acid. Furthermore, abnormally low levels of vitamin C have been found in the plasma and white blood cells of persons with pernicious anemia who have had an apparently adequate intake of vitamin C. The latter observation suggests excessive oxidative destruction or utilization of ascorbic acid in the pernicious anemia patient. To test this hypothesis, ascorbic acid and dehydroascorbic acid were determined in seven patients with pernicious anemia in severe relapse and in five control subjects without hematologic disease. Plasma specimens were obtained before the intravenous administration of 300 mg. of sodium ascorbate and fifteen minutes, one, two, and three hours afterward.

In the control group the fifteen-minute plasma specimens showed a prompt rise of ascorbic acid and a lesser rise of dehydroascorbic acid. Plasma specimens obtained at one, two, and three hours showed a fall in both ascorbic and dehydroascorbic acid approaching the preinjection levels.

In seven patients with pernicious anemia in severe relapse two showed plasma level curves of ascorbic and dehydroascorbic acid similar to the controls, and five showed a striking reversal. In the latter group the fifteen-minute blood specimens showed a prompt rise of dehydroascorbic acid and little or no rise of ascorbic acid. The plasma levels at one, two, and three hours showed a prompt fall in dehydroascorbic acid approaching preinjection levels and no rise in the ascorbic acid level. Following specific treatment normal relationships were reestablished.

These results indicate that in 5 or 7 pernicious anemia patients the normal relationship of ascorbic acid to its oxidation product, dehydroascorbic acid, is altered and that this alteration is corrected by specific therapy. The implications of these findings will be discussed.

160. CLINICAL EVALUATION OF DIPAXIN, AN ORAL ANTICOAGULANT

PARK W. WILLIS, III, M.D. (BY INVITATION), JACK A. MACRIS, M.D. (BY INVITATION), EDWARD W. DENNIS, M.D. (BY INVITATION), PAUL E. HODGSON, M.D. (BY INVITATION), WILLIAM W. COON, M.D. (BY INVITATION), JOHN R. GAMBLE, M.D. (BY INVITATION), AND IVAN F. DUFF, M.D., ANN ARBOR, MICH.

Dipaxin, 2-diphenylacetyl-l,2-diketo-hydrinene, an indandione derivative, has been administered orally to 104 individuals of whom 64 had normal pretreatment prothrombin values (Quick method). Comparison is made in Table I with patients treated previously with phenylindandione (PID), Dicumarol, and tromexan. The aim of therapy has been to maintain the prothrombin concentration between 20 and 30 per cent of normal.

TABLE I

	DIPAXIN	PID (PARKE, DAVIS & COMPANY)	DICUMAROL	TROMEXAN
Patients treated	64	133	122	50
Mean effective dose	63 mg.	685 mg.	646 mg.	3,120 mg.
Therapeutic effect achieved:				
Within 24 hr.	5%	18%	14%	12%
Within 48 hr.	67%	95%	55%	82%
Average (days)	2.3	1.3	2.3	2.1
Average days to recover to 40% or above	4.1	1.5	5.0	2.5
Incidence of prothrombin:				
Escape above 30%	38%	17%	37%	90%
Fall below 10%	10%	10%	15%	20%
Incidence of bleeding	11% (104 cases)	6.5% (200 cases)	11% (303 cases)	9% (100 cases)

The usual initial dose of Dipaxin was 30 mg. followed by 40 mg. in 3 doses over the next thirty-six hours; the average daily maintenance dose was 5 to 10 mg., given on a once or twice daily schedule. Dipaxin is the most potent of the oral anticoagulants studied. Two patients were observed in whom relatively large doses of Dipaxin had no significant effect upon prothrombin concentration; both subsequently exhibited a satisfactory response to phenylindandione. Bleeding was a complication in 11 per cent of the Dipaxin-treated patients; hypoprothrombinemia was quickly and efficiently reversed by intravenous or oral vitamin K_1. In two carefully studied individuals receiving Dipaxin there was no alteration in repeated renal function tests; albuminuria has not been observed.

161. THE ELECTROPHORETIC PATTERN OF SERUM PROTEINS IN ANAPHYLACTOID AND IDIOPATHIC THROMBOCYTOPENIC PURPURA

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KANSAS CITY, KAN.

During the past decade there has been considerable discussion about disturbances of blood clotting that are accompanied by an altered serum protein pattern. It has been demonstrated that the antihemophilic factor is

normal blood belongs to the globulins. In the hypoprothrombinemia (a globulin deficiency) of severe liver damage there is frequently a hyperglobulinemia. In the present study the electrophoretic pattern of the serum proteins was observed in 21 human subjects: 10 with anaphylactoid purpura, 9 with idiopathic thrombocytopenic purpura, and 2 subjects with thrombocytopenia and clinical anaphylactoid purpuric lesions. The filter paper method of electrophoresis was used in these studies.

In 4 of the 10 subjects with anaphylactoid purpura there was a marked elevation in the gamma globulin. Whether or not the hemorrhagic state was due to an abnormal gamma globulin is a controversial subject. Waldenström has referred to this disease or syndrome as purpura hyperglobulinemia. From our study the duration of the disease has no influence on the presence or absence of an increase in the gamma globulins.

Six of the 9 subjects with thrombocytopenic purpura were in a chronic state, 5 of whom were splenectomy failures. Three were of the acute type. The presence of an alpha-x globulin was not a constant finding in the electrophoretic pattern. Alpha-2 globulin was always absent.

The 2 subjects with thrombocytopenia and anaphylactoid lesions had no diagnostic electrophoretic pattern.

162. PROTEIN PATTERNS IN HUMAN GASTRIC JUICE AS IDENTIFIED BY THE ULTRACENTRIFUGE

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OKLAHOMA CITY, OKLA.

(INTRODUCED BY ROBERT GLASER, M.D.)

Pursuing earlier efforts to characterize the components of high molecular weight in human gastric juice we have analyzed in the ultracentrifuge the juice of normal subjects. Special attention was accorded to gentleness in handling the material in order to avoid, as far as possible, denaturation or other change in protein constituents.

Freshly collected gastric juice was transferred to a cold room at 4° C. where all of the procedures preparatory to ultracentrifugation were carried out. The insoluble constituents were spun off by preliminary centrifugation at 1,500 r.p.m. The clear supernatant solution was neutralized to pH 7, dialyzed against distilled water, and concentrated by evaporation to one-tenth the original volume. Samples of this material were then placed directly in the analytical cell of the Spinco ultracentrifuge and spun at 52,000 r.p.m. for ninety minutes.

The most characteristic pattern observed in healthy subjects included two distinct peaks which traversed the optical field in ninety and one hundred and twenty minutes, respectively. The faster of these was fairly symmetrical. Often a third, less well defined, more slowly moving peak was seen just separating from the point of origin at ninety minutes. The photographic records of typical patterns will be shown, together with simultaneously obtained records of the ultracentrifugal patterns of mucous substances precipitated from human gastric juice by the method of Glass and Boyd. The results of analysis of fractions partly separated by ultracentrifugation for total proteins and mucopolysaccharides will also be presented.

The findings of this study, carried out with very gentle procedures, confirm earlier studies done with cruder methods and indicate that several separate constituents of high molecular weight exist in native human gastric juice.

163. THE "DELAYED THERAPOCORTICOID EFFECT," A PREVIOUSLY UNDESCRIBED ACTION OF ADRENAL CORTICAL HORMONES. ITS IMPORTANCE FOR SUCCESSFUL TREATMENT OF EYE DISEASE

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(INTRODUCED BY GORDON B. MYERS, M.D.)

Long-acting corticotropin treatment individualized in dosage and duration regularly permits sustained remission in eye diseases reported to respond irregularly to hormones (Univ. Michigan Med. Bull. **18**: 1, 1952). Normal or useful vision has been restored and sustained without relapse in 89 per cent of 35 patients with acute chorioretinitis, acute optic or retrobulbar neuritis, acute central angiospastic retinopathy, and acute recurrent iridocyclitis refractory to topical cortisone. Similar results were obtained in 63 per cent of 30 patients with chronic chorioretinitis, chronic retrobulbar neuritis, and other chronic severe ocular disorders. In 15 patients of the latter group, recovery has appeared dependent upon a therapeutic effect of adrenal hormones fundamentally different from their usually prompt actions. After prolonged intensive treatment of acute disorders affecting one optic nerve or retina, some patients also showed slow improvement in severe old inactive "irreversible" lesions involving the other eye. With persistent treatment, normal or useful vision often was restored in both eyes without subsequent relapse. Subsequently, similar results were obtained in patients with only chronic "irreversible" neuroretinal disorders. At best, improvement begins slowly and is never dramatically sudden. Equally often, the beginning of improvement may not be unequivocally demonstrable until patients have been treated for from thirty to three hundred days. Maximum improvement usually requires treatment for one-half to over two years; the average patient has received 14,700 units of corticotropin during four hundred and fifty-five days. For the first hundred days, dosage averaged 69 units per day. Patients who are healthy except for localized eye disease appear relatively refractory to dangerous hypercorticoid manifestations. Slow progressive improvement, often preceded by prolonged therapeutic latency, constitutes the "delayed therapocorticoid effect," an action observed only in disorders of ocular neuroectodermal structures. Certain observations suggest its mechanism may involve anatomical restitution of structures which do not regenerate in eucorticoid individuals.

The Journal of Laboratory and Clinical Medicine

The Official Publication of the Central Society for Clinical Research

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PUBLISHED BY THE C. V. MOSBY COMPANY, 3207 WASHINGTON BLVD., ST. LOUIS 3, U. S. A.

Published monthly. Subscriptions may begin at any time.

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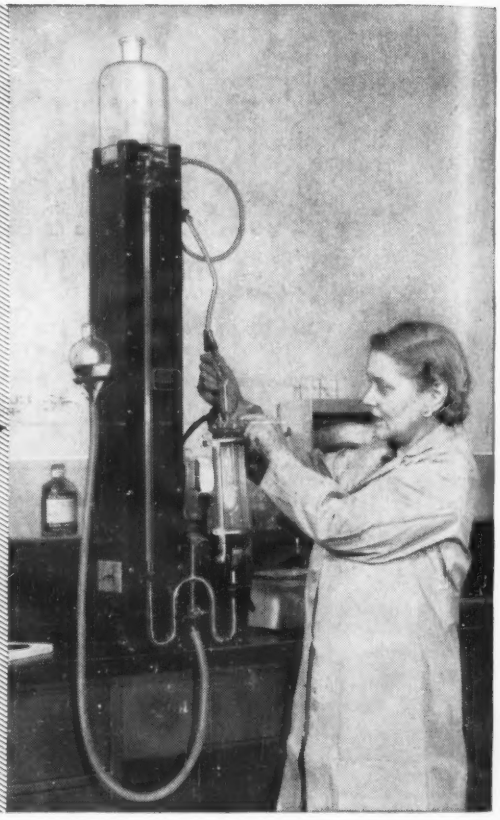

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